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NORADRENERGIC FUNCTION IN NK1+/+ AND NK1-/- MICE

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For those whom I love.

ABSTRACT

NK1 receptor antagonists represent an emerging class of putative antidepressants. These compounds may act through an interaction with 5-HT and / or noradrenaline (NA), the targets of established antidepressants. Pilot microdialysis studies in our laboratory have demonstrated that cortical NA efflux is 2-fold higher in halothane-anaesthetised NK1-/- mice compared with NK1+/+ mice. These current studies were, therefore, primarily aimed at investigating the autoregulatory α_2-adrenoceptor, which controls the firing-rate and release of NA from noradrenergic neurones.

NA efflux was monitored following systemic administration of the α_2-adrenoceptor antagonist RX821002, during anaesthetised (0.3 mg /kg i.p.), and freely-moving (0.3, 1.0 and 3.0 mg / kg i.p.), *in vivo* microdialysis. NA efflux and the behavioural response to an aversive, naturalistic stimulus were investigated using the light / dark exploration box (LDEB), in naive and RX821002 pre-treated mice. Localisation and density of α_2-adrenoceptors were examined using immunohistochemistry, Western blot analysis and [^{3}H]RX821002 autoradiography. Adrenaline-stimulated [^{35}S]GTPγS binding analysed the functional status of the α_2-adrenoceptors.

Basal NA efflux was 4-5-fold higher in halothane-anaesthetised NK1-/- mice compared with NK1+/+ mice. No difference in NA efflux between genotypes was found during freely-moving mouse microdialysis. Treatment with RX821002, increased NA efflux in NK1+/+ mice, only. Of the 12 behaviours scored in the LDEB, 5 were genotype dependent. With the exception of rearing activity, these were not dependent on the genotype difference in locomotor activity. In the LDEB pre-treatment with RX821002 modified the behavioural response in 4 of the 12 behaviours, and increased NA efflux in NK1+/+ mice, only. However, no difference in net NA efflux was found between groups. No difference between NK1+/+ and NK1-/- mice in the localisation, density or functional activity of the α_2-adrenoceptors was found.

These results suggest that genetic disruption of the NK1 receptor, modifies the regulation of the noradrenergic system, which can, at least in part, be attributed to a decreased sensitivity of the α_2-adrenoceptor.

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ABBREVIATIONS

AC	Adenylate cyclase
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
Benzodiazepines	BDZs
BNST	Bed nucleus of the stria terminalis
CBM	Cerebellum
ChP	Choroid plexus
Ci	Curie
CNS	Central nervous system
CRF	Corticotrophin releasing factor
DA	Dopamine
DβH	Dopamine-β-hydroxylase
DG	Dentate Gyrus
DMI	Desipramine
DR	Dorsal raphé
Ent	Entorhinal cortex
ECF	Extracellular fluid
FCtx	Frontal cortex
FITC	Fluorescein isothiocyanate
Fmol	Femtomole
FR2	Medial prefrontal cortex
GABA	γ-aminobutyrate
GIRKs	G protein-coupled inwardly rectifying potassium channel
GPCR	G protein-coupled receptor
Hipp	Hippocampus
5-HT	5-Hydroxytryptamine
HPLC / ECD	High performance liquid chromatography with electrochemical detection
i.c.v.	Intracerebroventricular
i.d.	Inner diameter
IHC	Immunohistochemistry
IgG	Immunoglobulin G

IL	Infralimbic cortex
i.p.	Intraperitoneal
kDa	Kilodalton
Kg	Kilogram
l	Litre
LC	Locus coeruleus
LDEB	Light dark exploration box
LSI	Lateral septum
M	Molar
M2	Secondary motor cortex
MCID	MicroComputer Imaging Device
Me5	Mesencephalic trigeminal nucleus
mg	Milligram
Min	Minute
µl	Microlitre
ml	Millilitre
µl	Microlitre
µM	Micromolar
µm	Micrometer
mm	Millimetre
mM	Millimolar
mmol	Millimole
µV	Microvolt
mV	Millivolt
MVe	Medial vestibular nucleus
NA	Noradrenaline
NAT	Noradrenaline transporter
NK1	Neurokinin1
nM	Nanomolar
NRI	Noradrenaline reuptake inhibitor
NSB	Non-specific binding
OB	Olfactory bulb
o.d.	Outer diameter
PAG	Periaqueductal grey

PB	Phosphate buffer
PCR	Polymerase chain reaction
PGi	Paragigantocellularis
PrH	Prepositus hypoglossi
RGS	Regulators of G protein signalling
SN	Substantia nigra
SP	Substance P
SPI	Subparafasicular thalamic nuclei
TH	Tyrosine Hydroxylase
TSA	Tyramide signal amplification
V	Volt

PUBLICATIONS ARISING FROM THIS THESIS

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Fisher AS, Stewart RJ, Hunt SP, & Stanford SC (2004). Investigation of the functional activity of alpha$_2$-adrenoceptors in NK1-/- and NK1+/+. *J Psychopharmacol* **18,** (Suppl): A71.

Fisher AS, Stewart RJ, Hunt SP, & Stanford SC (2004). Functional activity of alpha$_2$-adrenoceptors differs in NK1-/- and NK1+/+ mice. Program No. 1027.10. *Abstract Viewer and Itinerary Planner.* San Diego: Society for Neuroscience. Online.

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Fisher AS, Stewart RJ, Hunt SP, & Stanford SC (2005). Functional activity of alpha$_2$-adrenoceptors differs in NK1-/- and NK1+/+ mice. *British Neurosci Assoc* Abstr **18,** P11.

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CHAPTER ONE

INTRODUCTION

1 INTRODUCTION

Background

Depression is one of the most prevalent and costly brain disorders, with health and social effects similar to those for chronic diseases like hypertension, congestive heart failure or diabetes (WHO, 2005). Depression affects 5-10 % of the population of Western Europe (WHO, 2005), with 1 in 10 women and 1 in 50 men suffering at some stage in their life. It is a highly heritable disorder; genetic factors comprise roughly 50 % of the risk for depression (Nestler *et al.*, 2002). However, we still lack a detailed understanding of genes that increase risk for depression, and of the changes in the brain that may underlie the diverse symptoms of depression (for review see: (Merikangas *et al.*, 2002)).

Depression is often associated with co-morbid psychiatric disorders especially anxiety disorders. Chronic environmental stress / anxiety may also contribute to development of depressive states. Depression can, therefore, be viewed as a complex, polygenic disorder, arising from a complex interplay of environmental stress and developmental insults affecting multiple aspects of monoamine modulation.

Three main treatments are used: usually antidepressants, psychotherapy or electroconvulsive therapy (ECT). The main classes of established antidepressants act to increase the monoamines noradrenaline (NA) and / or serotonin (5-HT) in all brain areas. However, therapeutic lag before clinical efficacy, poor patient tolerance / compliance, the risk of overdose and faster efficacy, necessitates the development of new improved treatments.

Substance P (SP) belongs to the tachykinin family of neuropeptides, found throughout the central and peripheral nervous systems. It mediates many physiological responses for example those provoked by stress. The last ten years have seen dramatic progress in the development of potential new medicines targeting SP or its receptor. This is due, at least in part, to the cloning of the SP-preferring neurokinin 1 (NK1) receptor (Yokota *et al.*, 1989).

NK1 receptor antagonists are emerging as a class of putative anxiolytic / antidepressant compounds (Kramer *et al.,* 2004). Unlike established antidepressants, which target the monoamines 5-HT and / or NA in all brain areas, NK1 receptor antagonists act by inhibiting the actions of the neuropeptide SP within the central nervous system (CNS). Although, it should also be noted that phase III clinical trials with the NK1 receptor antagonist, aprepitant, demonstrate aprepitant is not more effective than placebo (Kramer *et al.,* 2004).

It is possible that NK1 receptor antagonists produce behavioural effects via modulation of central noradrenergic and / or serotonergic neurotransmission. Previous studies in other laboratories have focused on the role of NK1 receptors in modulating serotonergic neurotransmission (Froger *et al.*, 2001; Santarelli *et al.*, 2001).

The locus coeruleus (LC) is the main source of central noradrenergic nerve terminals (Dahlstrom and Fuxe, 1964; Aston-Jones *et al.,* 1986). LC neurones are innervated by SP containing fibres (Halliday *et al.*, 1988), express NK1 receptors (Shults *et al.*, 1984) and are activated by SP (Cheeseman *et al.*, 1983). Investigations into the mechanism of action of NK1 receptor antagonists are also beginning to focus on the effects of SP on the activity of noradrenergic neurones (Haddjeri & Blier, 2000; Maubach *et al.*, 2002), therefore. The studies contained in this thesis are, particularly focused on the role the NK1 receptor may play in modulating noradrenergic neurotransmission.

1.1 TACHYKININS AND THEIR RECEPTORS

Three main tachykinin peptides are recognised; substance P (SP), neurokinin A (NKA) and neurokinin B (Nakanishi *et al.*, 1993), characterized by a common C-terminal amino acid sequence (for review see: (Otsuka & Yoshioka, 1993)). Each binds specifically and with different affinities to the NK1, NK2 or NK3 receptors, respectively, which differ in respect of their distribution throughout the CNS (Pinto *et al.*, 2004).

1.1.1 Distribution of the NK1 receptor

Extensive distribution of the NK1 receptor is found throughout the central nervous system (CNS). Heavy expression is found in the olfactory bulbs, the striatum, the amygdala, the habenula, the periaqueductal grey (PAG), the superior colliculus, the parabrachial nucleus and the LC (Gadd, 2004). The NK1 receptor is also found in high levels in the superficial laminae of the spinal cord, where it is expressed on neurones that are activated by SP released from nociceptive C-fibres (Nakaya *et al.*, 1994).

1.1.2 Signalling through the NK1 receptor

The undecapeptide SP [Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH2] was initially isolated in the 1930s (von Euler and Gaddum 1931). The release of SP upon neuronal excitation evokes a number of cellular responses, mediated by activation of the NK1 receptor. Tachykinins usually evoke a slow long-lasting membrane depolarisation in neurones and smooth muscle fibres which is excitatory and often accompanied by a fast excitatory post-synaptic potential. The evoked cellular responses include blockade of an inwardly rectifying K^+ channel, resulting in membrane depolarisation and an increased excitability of the neurones (Nicoll, 1978; Nowak & Macdonald, 1982; Stanfield *et al.*, 1985; Yamaguchi *et al.*, 1990; Shen & North, 1992).

The main signalling pathway activated by SP and related tachykinins is activation of G proteins ($G_{q/11}$, $G_{\alpha s}$ and $G_{\alpha o}$) mediating inositol phospholipid hydrolysis via phospholipase C_β activation and formation of diacylglycerol. Inositol phospholipid hydrolysis yields inositol phosphates, e.g. 1,4,5-trisphosphate (IP_3) (Taylor *et al.*, 1986; Nakajima *et al.*, 1992; Takeda *et al.*, 1992; Seabrook & Fong, 1993; Roush & Kwatra, 1998). IP_3 triggers release of Ca^{2+} from internal stores and the subsequent activation of calcium-calmodulin-dependent kinases. IP_3 may also promote Ca^{2+} influx, leading to elevated $[Ca^{2+}]_i$, while DAG activates protein kinase C (Merritt & Rink, 1987; Sugiya *et al.*, 1987; Womack *et al.*, 1988; Seabrook & Fong, 1993).

1.1.3 Distribution of Substance P in the central nervous system

SP-immunoreactive cell bodies and terminal networks are found in most areas of the brain (Ljungdahl *et al.*, 1978a), in close association with 5-HT and NA-containing neurones (Ljungdahl *et al.*, 1978b; Hokfelt *et al.*, 1987) the target of all established antidepressant drugs (Figure 1.1).

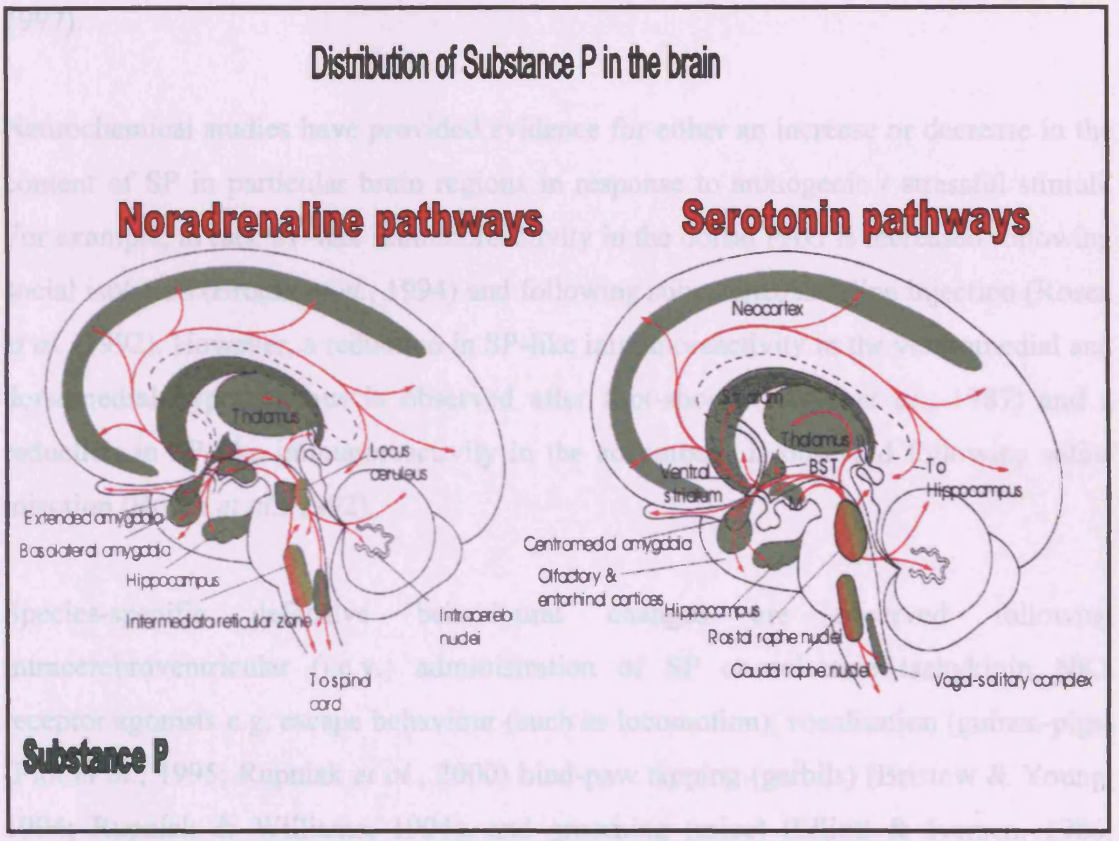


FIGURE 1.1: Distribution of the SP pathways in close association with the 5-HT and noradrenaline pathways in the brain.

Structures within the limbic system, which governs mood and cognition, express a high density of NK1 receptors. These brain structures include the striatum, hippocampus, nucleus tractus solitarius, raphé nuclei and LC (Otsuka & Yoshioka, 1993). Areas such as the amygdala, septum, hippocampus, hypothalamus and periaquaductal grey (PAG), involved in 'fear processing', also express SP and NK1 receptors (Mantyh *et al.*, 1984; Arai & Emson, 1986). On this basis, it can be speculated that SP may influence limbic behaviours such as affective responses.

1.1.4 Substance P and aversive / stressful stimuli

Noxious / stressful stimuli are associated with changes in the release of SP in a region-specific manner. Evidence, from studies on rodents, suggests that following noxious stimulation for example heating, tail pinch or cold stimulation, SP synthesis and release is increased within areas of the CNS such as the dorsal root ganglion (Noguchi *et al.*, 1988), substantia gelatinosa (Lang & Hope, 1994), and PAG (Xin *et al.*, 1997).

Neurochemical studies have provided evidence for either an increase or decrease in the content of SP in particular brain regions in response to anxiogenic / stressful stimuli. For example, in rats, SP-like immunoreactivity in the dorsal PAG is increased following social isolation (Brodin *et al.*, 1994) and following subcutaneous saline injection (Rosen *et al.*, 1992). However, a reduction in SP-like immunoreactivity in the ventromedial and dorsomedial hypothalamus is observed after foot-shock (Siegel *et al.*, 1987) and a reduction in SP-like immunoreactivity in the accumbens is observed following saline injection (Rosen *et al.*, 1992).

Species-specific defensive behavioural changes are observed following intracerebroventricular (i.c.v.) administration of SP or selective tachykinin NK1 receptor agonists e.g. escape behaviour (such as locomotion), vocalisation (guinea-pigs) (Piot *et al.*, 1995; Rupniak *et al.*, 2000) hind-paw tapping (gerbils) (Bristow & Young, 1994; Rupniak & Williams, 1994), and grooming (mice) (Elliott & Iversen, 1986; Elliott *et al.*, 1992). I.c.v. infusion of SP produces 'anxiety-like' behaviours in a range of tests used to screen anxiolytic and antidepressant compounds e.g. the elevated plus-maze (Aguiar & Brandao, 1996; Teixeira *et al.*, 1996), conditioned place aversion (Aguiar & Brandao, 1994) and aggression (Shaikh *et al.*, 1993). Cardiovascular changes, specifically resembling the defence response to threatening stimuli (Culman & Unger, 1995), are also reported.

The medial amygdala is crucial for the mediation of SP transmission in anxiety-like responses (Ebner *et al.*, 2004). Aversive stimuli such as immobilization stress, elevated plus-maze exposure and maternal separation result in the release of SP in the amygdala, and NK1 receptor antagonism prevents the vocalisations associated with these stimuli

(Kramer *et al.*, 1998; Smith *et al.*, 1999; Boyce *et al.*, 2001; Steinberg *et al.*, 2002; Ebner *et al.*, 2004). These behavioural and physiological effects suggest that SP is released in response to noxious / aversive stimulation.

1.1.5 Behavioural and neurochemical effects of NK1 receptor antagonists

A number of reports support the anxiolytic / antidepressant profile of NK1 receptor antagonists in a variety of pre-clinical behavioural tests (Table 1.1).

NK1 receptor antagonist	Behavioural test	Species	Effect	Reference:
FK888	Elevated plus-maze	Mice	Anxiolytic	(Teixeira *et al.*, 1996; Teixeira & De Lima, 2003)
		Rats		(Duarte *et al.*, 2004; Gavioli *et al.*, 2002)
	Forced swim test	Rats	Reduces immobility	(Dableh *et al.*, 2005).
NKP608	Social interaction test	Rats	Anxiolytic	(File, 2000)
	Chronic mild stress	Rats	Antidepressant	(Papp *et al.*, 2000)
CGP49823	Social interaction test	Rats	Anxiolytic	(File, 1997)
L-760735	Social interaction test	Gerbils	Anxiolytic	(Cheeta *et al.*, 2001)

TABLE 1.1: Anxiolytic / antidepressant profile of NK1 receptor antagonists in behavioural tests used to determine anxiolytic / antidepressant activity.

The compound MK869, its close analogue L-760735, and a structurally unrelated compound, L-733060, have been studied in a range of preclinical tests. These compounds completely inhibited stress-induced vocalisations in guinea-pig pups induced by transient separation from their mother (one of a number of behavioural assays used to detect antidepressant compounds) (Kramer *et al.*, 1998). The NK1 selectivity of this response was confirmed by the use of the enantiomers L-770765 and

L-733061, which have low affinity for the NK1 receptor; these compounds produced no inhibition of vocalisation. The results were confirmed using established antidepressant drugs, fluoxetine (a serotonin re-uptake inhibitor, SSRI) and imipramine (a tricyclic antidepressant), which also markedly decreased separation-induced vocalisations in guinea-pigs. It was these observations that led to the first clinical investigation of the NK1 receptor antagonist MK869 in depressed patients (Kramer *et al.*, 1998; Rupniak & Kramer, 1999). In humans, MK869 was shown to alleviate major depressive disorder with moderately high anxiety (Kramer *et al.*, 1998).

However, preclinical evaluation of NK1 receptor antagonists, using traditional screens for antidepressant drugs in rodents, is complicated by marked species differences in NK1 receptor pharmacology (Gitter *et al.*, 1991; Beresford *et al.*, 1991)(Table 1.2) and poor brain penetration (Rupniak *et al.*, 1997). Poor brain penetration is overcome by administration of compounds at high doses. However, this results in non-specific ion channel effects, conferring activity in *in vivo* assays independent of NK1 receptor blockade (Rupniak *et al.*, 1993; Rupniak & Jackson, 1994).

Development of the NK1-/- mouse has provided a valuable research tool (De Felipe *et al.*, 1998). The NK1-/- mouse has enabled investigation into the contribution made by the NK1 receptor, in adaptive responses to stress, in a species for which there are, as yet, no suitable pharmacological antagonists. However, compensatory effects, resulting from disruption of the receptor, must always be kept in mind.

NK1 receptor antagonist	Species	Binding affinity (nM)	Reference:
RP67580	Rat	7.9	(Barr & Watson, 1993)
RP67580	Human	194	(Barr & Watson, 1993)
CP-96,345	Rat	210	(Barr & Watson, 1993)
CP-96,345	Human	0.99	(Barr & Watson, 1993)
CP-96,345	Cow	0.6	(Gitter *et al.*, 1991)
CP-96,345	Guinea-pig	0.32	(Gitter *et al.*, 1991)
CP-96,345	Rabbit	0.54	(Gitter *et al.*, 1991)
CP-96,345	Mouse	32	(Gitter *et al.*, 1991)
CP-96,345	Rat	35	(Gitter *et al.*, 1991)
CP-96,345	Chicken	156	(Gitter *et al.*, 1991)
Spantide	Rat	602	(Fardin & Garret, 1991; Appell *et al.*, 1992)
Spantide	Guinea-pig	247	(Fardin & Garret, 1991; Appell *et al.*, 1992)
L688169	Rat	602	(Fardin & Garret, 1991)
L688169	Guinea-pig	247	(Fardin & Garret, 1991)

TABLE 1.2: Species differences in binding affinity of NK1 receptor antagonists.

1.1.6 The NK1-/-mouse has an antidepressant / anxiolytic-like phenotype

Behavioural and neurochemical analysis of NK1-/- mice has revealed a number of features consistent with chronic treatment with an antidepressant.

1.1.6.1 Anxiolytic-like behavioural phenotype

The NK1-/- mouse shows a marked reduction in aggressive behaviour in the resident intruder test and a decrease in separation-induced neonatal vocalisation (De Felipe *et al.*, 1998; Rupniak *et al.*, 2000; Santarelli *et al.*, 2001; Rupniak *et al.*, 2001) compared to its wildtype counterpart. Both these behaviours are modified in NK1+/+ mice, by antidepressants e.g. fluoxetine, in such a way that NK1+/+ mice are indistinguishable from the NK1-/- mice. An increase in exploratory behaviour in the open field, a

reduction in anxiety-like behaviour in the elevated plus-maze, novelty suppressed feeding (Santarelli *et al.*, 2001; Santarelli *et al.*, 2002) and in the forced swim test (Rupniak *et al.*, 2001) are also reported by certain laboratories using NK1-/- mice bred with a 129/SvEv background. However, no difference between genotypes in the open field, novel object exploration and elevated plus-maze was found by De Felipe *et al.*, (1998) and Rupniak *et al.*, (2001) using NK1-/- mice derived from hybrid 129/SvEv X C57BL/6 mice. However, both these groups do report a reduction in aggression in the resident intruder test in NK1-/- mice (Rupniak *et al.*, 2001; De Felipe *et al.*, 1998). Strain differences of the NK1-/- mice could, therefore, account for the discrepancies in the behavioural response of these two groups.

1.1.6.2 Increased neurogenesis

The long time course needed to increase neurogenesis and proliferation resembles the delayed time course needed before the therapeutic action of antidepressants is apparent. Electroconvulsive shock treatment (ECT), another treatment for depression, increases neurogenesis in the adult rat (Madsen *et al.*, 2000; Malberg *et al.*, 2000; Hellsten *et al.*, 2002). Consistent with an antidepressant-like phenotype, the NK1-/- mouse shows an increase in neurogenesis in the dentate gyrus of the hippocampus (Morcuende *et al.*, 2003). Brain derived neurotrophic factor (BDNF) protein has been shown to have antidepressant-like effects when injected into the hippocampus (Shirayama *et al.*, 2002; Sairanen *et al.*, 2005) and antidepressant treatment can result in increased BDNF (Chen *et al.*, 2001). NK1-/- mice show a 2-fold increase in BDNF compared to NK1+/+ mice in the hippocampus (Morcuende *et al.*, 2003). The 29.3 % increase in neurogenesis in the dentate gyrus of NK1-/- mice, is approximately the same as that seen in rats treated with an SSRI (Malberg *et al.*, 2000).

The monoamines 5-HT and NA both play an important role in regulating neurogenesis (for a review see: (Delgado, 2004; Marien *et al.*, 2004)). Depletion of NA, using the noradrenergic neurotoxin DSP-4, decreases proliferation, but not survival and differentiation of cells, in the adult rat hippocampus (Kulkarni *et al.*, 2002). Thus there may be an interaction between the monoamine systems and NK1 receptors in controlling neurogenesis.

1.1.6.3 Downregulation and desensitisation of 5-HT$_{1A}$ autoreceptors

Previous studies, by other groups, have demonstrated that either disruption of the NK1 receptor or, administration of an NK1 receptor antagonist, results in profound changes in the regulation of the serotonergic system, suggesting that NK1 receptor antagonists exert their therapeutic effects through modulation of the serotonergic system.

The 5-HT$_{1A}$ receptor has an inhibitory influence on the firing-rate of dorsal raphé (DR) serotonergic neurones, and hence release of 5-HT in terminal projection areas, for example, the hippocampus. Chronic antidepressant treatment results in downregulation of the autoregulatory 5-HT$_{1A}$ receptor (Blier & de Montigny, 1983; Jolas *et al.*, 1994; Le *et al.*, 1995). The 5-HT$_{1A}$ receptor in the NK1-/- mouse is also downregulated and desensitised (Froger *et al.*, 2001; Santarelli *et al.*, 2001). Administration of a selective serotonin re-uptake inhibitor (SSRI) e.g. paroxetine, results in an increase in 5-HT, however, this increase is hampered by the autoregulatory 5-HT$_{1A}$ receptor. In NK1-/- mice, therefore, downregulation / desensitisation of the 5-HT$_{1A}$ receptor results in an enhancement of 5-HT release following challenge with the selective serotonin re-uptake inhibitor (SSRI) paroxetine compared to their wildtype counterpart (Froger *et al.*, 2001; Santarelli *et al.*, 2001).

Although several studies support modulation of the serotonergic system in the anxiolytic / antidepressant mechanism of action of NK1 receptor antagonists, we are particularly interested in the contribution made by the noradrenergic system, in mediating the therapeutic actions of NK1 receptor antagonists.

1.2 NORADRENALINE IS IMPLICATED IN ANXIETY / DEPRESSION

A major difficulty in unravelling the neurobiological basis of depression is that it is not a simple, unitary disorder (Stanford, 2001c). Although the role of monoamines, such as NA and 5-HT, in mood disorders has long been recognised, (for review see: (Ressler & Nemeroff, 1999; Millan, 2004), the precise nature of any defects remains equivocal , and no consistent biochemical markers have emerged to provide a firm link (Table 1.3).

Marker	Tissue	Usual finding in depression	
NA or MHPG concentrations	Post-mortem brain, urine, CSF, plasma	NCC	
Tyrosine hydroxylase immunoreactivity	Post-mortem brain	NCC	
α_1-adrenoceptor binding	Post-mortem brain	↓	Some brain areas
α_2-adrenoceptor binding	Post-mortem brain	↑	High-affinity site in some brain areas
β_1-adrenoceptor binding	Platelets Post-mortem brain	NCC ↓	Certain cortical areas of suicide victims
β_2-adrenoceptor binding –cAMP response	Lymphocytes	↓ ↓	

TABLE 1.3: Neurochemical markers for depression. Measurements in depressed patients compared with normal subjects, euthymic controls or patients suffering from unrelated psychiatric disorder. NCC: No consistent change. (Taken from Stanford, 2001a).

More convincing evidence is available for a link between monoaminergic transmission and the therapeutic effects of antidepressants. Depletion of NA (by administration of the NA synthesis inhibitor, α-methyl-*p*-tyrosine) demonstrates the importance of functionally competent monoaminergic pathways for treating depressive states (Delgado, 2000). Depletion of NA causes a recurrence of depression in patients who are in remission following treatment with antidepressants that selectively target noradrenergic neurones (Stanford, 2001a).

The LC contains the majority of noradrenergic neurones projecting to forebrain regions (Dahlstrom and Fuxe, 1964). The LC-noradrenergic system is important in activation of the autonomic nervous system (Elam *et al.*, 1986b; Elam *et al.*, 1986a; Reiner, 1986; Svensson, 1987; Guo *et al.*, 2002). In the CNS it plays an important role in arousal, selective attention (Delagrange *et al.*, 1993; Aston-Jones *et al.*, 1994; Aston-Jones *et al.*, 2001; Jones, 2003), mood, (Osaka & Matsumura, 1994; Kayama & Koyama, 1998; Kayama & Koyama, 2003), and pain regulation (Stenberg, 1989; Jones, 1991), states which are profoundly altered in depressive disorder.

All antidepressant treatments alter the noradrenergic system in some way, demonstrating that monoamine systems are integral to the mechanism of action of antidepressants. Most classes of antidepressants restore compromised activity of corticolimbic noradrenergic pathways by 1) blockade of pre-synaptic noradrenaline transporter proteins, which sequester extracellular transmitter 2) inhibition of monoamine oxidase, which degrades monoamine neurotransmitters and 3) inhibition or excitation of pre-synaptic receptors that regulate monoamine transmitter release and / or firing-rates and so increase release. Treatment with antidepressants results in significant alteration of NA neuronal activity and alteration of noradrenergic receptors. It is these alterations that are believed to mediate recovery from depression. Alterations may arise from changes in neurotrophin release and synaptic plasticity, or through altered interactions of the brain stem modulatory systems. In a similar manner to the 5-HT_{1A} receptors, the expression of α_2-adrenoceptors is also changed following antidepressant treatment. ECT leads to downregulation of α_2-adrenoceptors (Stanford & Nutt, 1982). The effect of chronic antidepressant treatment on α_2-adrenoceptors is more variable, with some groups reporting an increase (Reisine., 1980), a decrease (Smith *et al.*, 1981), or no effect, depending on the brain region studied (see: (Stanford *et al.*, 1983)). Since the 5-HT_{1A} autoreceptor in NK1-/- mice is downregulated / desensitised resembling treatment with an SSRI, it is, therefore, also possible that the α_2-adrenoceptor in NK1-/- mice may be altered in someway.

Although the protein targets of antidepressants are known and their effects occur within the CNS almost immediately, the therapeutic lag means that clinical effects are not significantly different from placebo treated controls for at least 3-5 weeks. This suggests

slow adaptive processes downstream of structures, within the limbic system, that receive monoaminergic projections.

1.3 LOCUS COERULEUS: THE MAIN SOURCE OF NORADRENALINE AND ITS IMPLICATION IN DEPRESSION

1.3.1 Topography and efferent projections

Although the noradrenergic system of the rat has been extensively mapped (Foote *et al.*, 1980a; Loughlin *et al.*, 1982; Loughlin *et al.*, 1986b; Loughlin *et al.*, 1986a; Maeda *et al.*, 1991; Iijima, 1993), little is known about the distribution of noradrenergic axons and terminals within the mouse CNS. The brainstem contains the soma of noradrenergic neurones localised bilaterally in seven groups (A1-A7; Figure 1.2).

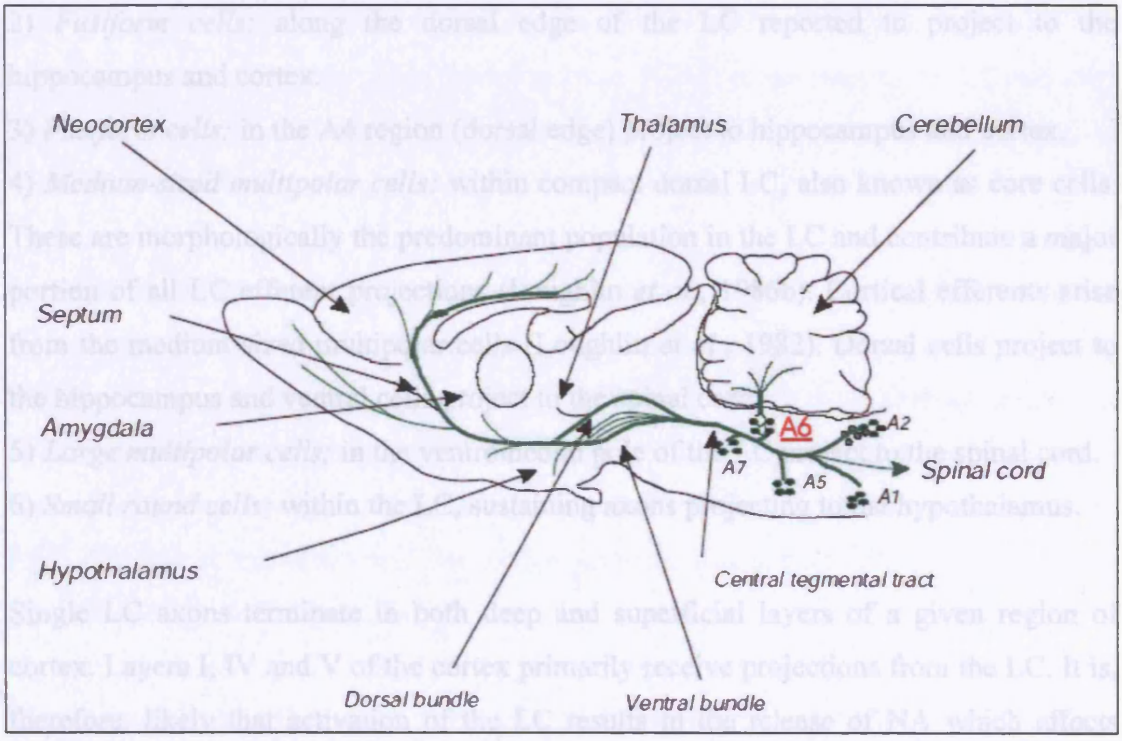


FIGURE 1.2: Distribution of noradrenergic neurones in the rat brain. The cell bodies are clustered in nuclei (A1-A7) in the pons / medulla regions of the brainstem and their axons project both rostrally and caudally to most regions of the neuraxis. The major nucleus is the locus coeruleus (A4 and A6) (Taken from Stanford, 2001b).

The LC (catecholaminergic cell group A4 and A6) of the rat brain stem, supplies 40 % of the total noradrenergic neurones in the CNS. LC projections innervate most of the cortical and subcortical areas in addition to the spinal cord (Figure 1.2). The LC is generally viewed as a widely projecting, topographically organised afferent system (see: (Ressler & Nemeroff, 1999)).

Using 3-D reconstruction of the LC it has been shown that the LC consists of a densely packed globular core and three extended poles: an anterior pole, a ventral cell group and the A4 group projecting dorso-posteriorly from the LC (Loughlin *et al.*, 1982). Six morphologically defined subpopulations of LC cells exist, differing in localization, morphology and / or efferent projections. The following morphological and spatial organisation of the LC in the rat has been proposed by (Loughlin *et al.*, 1986b):

1) *Anterior large multipolar cells*; these are believed to project to the hypothalamus.
2) *Fusiform cells;* along the dorsal edge of the LC reported to project to the hippocampus and cortex.
3) *Fusiform cells;* in the A4 region (dorsal edge) project to hippocampus and cortex.
4) *Medium-sized multipolar cells;* within compact dorsal LC, also known as core cells. These are morphologically the predominant population in the LC and contribute a major portion of all LC efferent projections (Loughlin *et al.*, 1986b). Cortical efferents arise from the medium sized multipolar cells (Loughlin *et al.*, 1982). Dorsal cells project to the hippocampus and ventral cells project to the spinal cord.
5) *Large multipolar cells;* in the ventromedial pole of the LC project to the spinal cord.
6) *Small round cells;* within the LC, sustaining axons projecting to the hypothalamus.

Single LC axons terminate in both deep and superficial layers of a given region of cortex. Layers I, IV and V of the cortex primarily receive projections from the LC. It is, therefore, likely that activation of the LC results in the release of NA which affects multiple, functionally distinct areas (Loughlin *et al.*, 1982).

1.3.2 Afferent projections to the LC

The LC is innervated by neurones from only a few brain regions. A dense plexus of LC neuronal processes, primarily dendrites, extend beyond the borders of the LC forming

the pericoerulear region (Shipley *et al.*, 1996) which receives input from a greater number of brain regions. The bulk of afferents to the LC complex (A6 and A4) arise from two rostral medullary nuclei, particularly important in driving LC neuronal firing (Aston-Jones *et al.*, 1986; Pieribone & Aston-Jones, 1988), (for review see: (Aston-Jones *et al.*, 1991b; Williams *et al.*, 1991; Berridge & Waterhouse, 2003)) .

One, an excitatory pathway mediated by the amino acid glutamate, arises from the nucleus paragigantocellularis (PGi) (Ennis & Aston-Jones, 1988). The other, an inhibitory pathway, arises from GABAergic neurones from the prepositus hypoglossi (PrH) (Ennis & Aston-Jones, 1989). The LC may also receive an inhibitory noradrenergic input thought to arise from dendrites of LC neurones themselves and via a noradrenergic inhibitory collateral input (Aghajanian *et al.*, 1977; Cedarbaum & Aghajanian, 1978).

The PGi participates in somatic, autonomic, and visceral functions. It is also the principal source of corticotrophin releasing factor (CRF) projections to the LC and may play a key role in the interaction between the stress response and NA leading to systemic arousal.

The PrH integrates velocity-coded saccadic inputs (Goldman *et al.*, 2002) to drive horizontal eye movements, supporting a role for this nucleus in the modulation of attentional changes that accompany visual shifts. Additional input sources to the LC arise from areas that participate in the mediation of the stress response and autonomic function, e.g. the paraventricular nucleus, the intermediate zone of the spinal cord, the PAG, the lateral hypothalamus, the medial preoptic area and the pericoerulear region (see: (Singewald & Philippu, 1998)).

The pericoerulear region is the target of a large number of pre-synaptic fibres, arising from the prefrontal cortex, central nucleus of the amygdala, lateral hypothalamus, bed nucleus of the stria terminalis (BNST) and DR (Pickel *et al.*, 1977; Arnsten & Goldman-Rakic, 1984; Peyron *et al.*, 1998; Van Bockstaele *et al.*, 1996b; Van Bockstaele *et al.*, 1996a; Van Bockstaele *et al.*, 1998; Van Bockstaele *et al.*, 1999a; Van Bockstaele *et al.*, 1999b). In addition, the LC receives an inhibitory input from the frontal cortex (Arnsten & Goldman-Rakic, 1984; Sara & Herve-Minvielle, 1995). This

provides further support for a role of the NA system in integrating the peripheral and central responses to stress and arousal.

LC noradrenergic neurones fire spontaneously. Under basal conditions, LC neurones fire action potentials asynchronously but, in response to a strong synaptic input, they fire in synchrony (Aston-Jones & Bloom, 1981b; Ennis *et al.*, 1992). Electrotonic coupling exists among the noradrenergic neurones of the LC (Ishimatsu & Williams, 1996), particularly between dendrites of the pericoerulear region, and it is believed that these electrical interactions between the dendrites of the pericoerulear region could lead to the synchronous activity within the nucleus (Ishimatsu & Williams, 1996).

LC neurones can be activated by a wide variety of noxious and non-noxious sensory stimuli (Foote *et al.*, 1980b) and are actively inhibited during REM sleep (Aston-Jones & Bloom, 1981a). Neurones of the LC may, therefore, weigh input from the major afferent inputs, and widely distribute a uniform message over its divergent efferents (Aston-Jones *et al.*, 1986).

1.3.3 NK1 receptor antagonists increase activity of the locus coeruleus

Noradrenergic neurones of the LC demonstrate both immunoreactivity (Chen *et al.*, 2000) and contain mRNA (Maeno *et al.*, 1993) for the NK1 receptor. The LC is densely innervated by SP-containing fibres (Ljungdahl *et al.*, 1978b). Electron microscopy shows that SP-containing axon terminals form asymmetric axodendritic synapses with catecholamine-containing neurones in the LC (Pickel *et al.*, 1979). As yet, there is a lack of direct evidence reporting the origin of SP afferents to the LC. However, one study does demonstrate that a single LC noradrenergic neurone forms both symmetrical synapses from GABAergic afferents and asymmetric synapses from SPergic afferents (Tamiya, *et al.*, 1994). It is possible that SP containing afferents arise from either the PGi (Dean *et al.*, 1993) or the PrH the main afferent projection areas to the LC. Direct application of SP to the LC of an anaesthetised rat, or *in vitro* slice preparations, increases the firing-rate of most cells in the LC (Guyenet & Aghajanian, 1977; Cheeseman *et al.*, 1983) through NK1 receptors expressed on LC neurones.

However, dialysis and electrophysiological studies have shown that NK1 receptor antagonists also increase cortical NA efflux, and increase the firing-rate of LC noradrenergic neurones. Treatment with the NK1 receptor antagonist L-760735, induced burst-firing of neurones in the guinea-pig LC (Maubach *et al.*, 2002). The selective NK1 receptor antagonist GR205171 dose dependently elevated cortical NA efflux in freely-moving rats (Millan *et al.*, 2001) and increased the firing-rate of noradrenergic cells in the LC of anaesthetised rats (Millan *et al.*, 2001). However, a single intravenous injection of the NK1 receptor antagonists WIN51, 708 and CP-96, 345 did not modify the spontaneous firing-rate of noradrenergic neurones in rats (Haddjeri & Blier, 2000). However, WIN51, 708 and CP-96, 345 attenuated the suppressant effect of the α_2-adrenoceptor agonist clonidine, so the authors concluded that NK1 receptor antagonists markedly affect the noradrenergic system via attenuation of the α_2-adrenoceptors on the cell bodies of noradrenergic neurones (Haddjeri & Blier, 2000), providing further evidence to suggest that α_2-adrenoceptors may be different between NK1+/+ and NK1-/- mice. Available evidence, therefore, suggests that blockade of NK1 receptors may facilitate the activity of noradrenergic pathways in the LC via attenuation of the activity of the α_2-adrenoceptors (Haddjeri & Blier, 2000) or an increase in firing-rate of noradrenergic neurones (Millan *et al.*, 2001; Maubach *et al.*, 2002).

Although nothing is known about the firing-rate of LC neurones in NK1-/- mice, the effect of NK1 receptor disruption and NK1 receptor antagonism on the firing-rate of serotonergic DR neurones is well documented. These studies report either no difference in basal firing-rate of DR neurones (Froger *et al.*, 2001), or a higher basal firing-rate in NK1-/- mice (Santarelli *et al.*, 2001). The reason for this discrepancy in the basal firing rate could be due to different strains of mice used by each group. Froger *et al.*, (2001) used 129/SvEv NK1-/- mice whereas Santerelli *et al.*, (2001) used NK1-/- mice derived from a pure 129/SvEv background.

The close association of SP with noradrenergic neurones, the high expression of NK1 receptors on LC neurones, and the effect of NK1 receptor antagonists on LC noradrenergic neurones, has prompted these current studies to investigate the effect of NK1 receptor disruption on noradrenergic neurones.

1.3.3.1 Noradrenaline efflux is higher in NK1-/- mice

Pilot microdialysis studies conducted in this laboratory have demonstrated a 2-5 fold difference in cortical basal NA efflux between halothane-anaesthetised NK1+/+ and NK1-/- mice (Fisher *et al.*, (2003)., Stewart *et al.*, (2004)., Herpfer *et al.*, (2005)). The studies contained in this thesis, are, therefore, aimed at investigating possible mechanisms that may underlie this difference in NA efflux.

1.4 INDICES OF NORADRENERGIC DYSREGULATION

1.4.1 In vivo microdialysis coupled with HPLC / ECD measurement of noradrenaline efflux

Microdialysis is an *in vivo* sampling technique, used extensively to assess the neuropharmacodynamic profile of drugs (see Sections 2.4-2.6 for a more detailed description of the technique). The technique continuously samples endogenous solutes, in the extracellular fluid of a specific tissue, in restrained, anaesthetised or freely-moving animals. Microdialysis, therefore, measures changes in 'efflux' of solutes harvested through probes. Efflux reflects the net effect of release and re-uptake on neurotransmitter concentration, providing an indication of the approximate concentration of solutes in the extracellular fluid.

The basic tool for sampling in microdialysis is the microdialysis probe. The probe is introduced to the tissue of interest, and perfused with a liquid which stabilises with the fluid outside the membrane by diffusion in both directions (Figure 1.3).

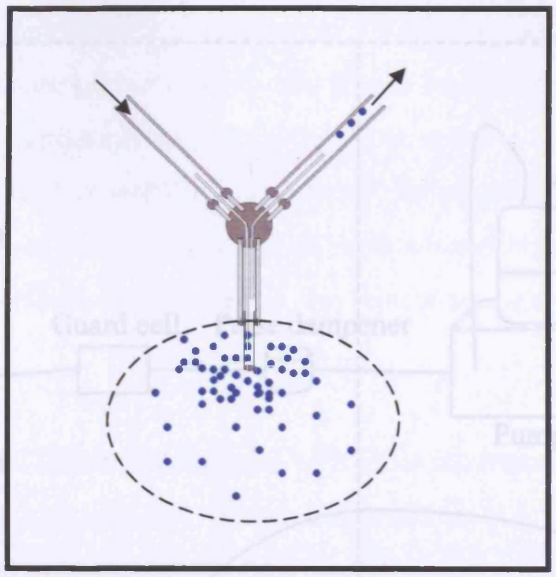


FIGURE 1.3: Diagram of the probe the basic tool used in these studies. The probe has direct access to the interstitial fluid compartment; the solute of interest diffuses into the probe down its concentration gradient. Adapted from (Benveniste & Huttemeier, 1990).

The average pore size of the probe is large enough (5-20 kDa molecular weight cut-off) to allow free diffusion of solute molecules, but small enough to resist diffusion of proteins and other macromolecules. The dialysate is collected from the outlet of the probe and then injected directly into the analytical system. In these studies sample analysis was performed using HPLC / ECD (Figure 1.4).

This technique has demonstrated that halothane-anaesthetised NK1-/- mice possess a 2-5 fold higher basal NA efflux compared to their NK1+/+ counterparts. For this reason the studies contained in this thesis are aimed at investigating possible candidates that may contribute to, or be the cause of, this higher basal NA efflux in NK1-/- mice.

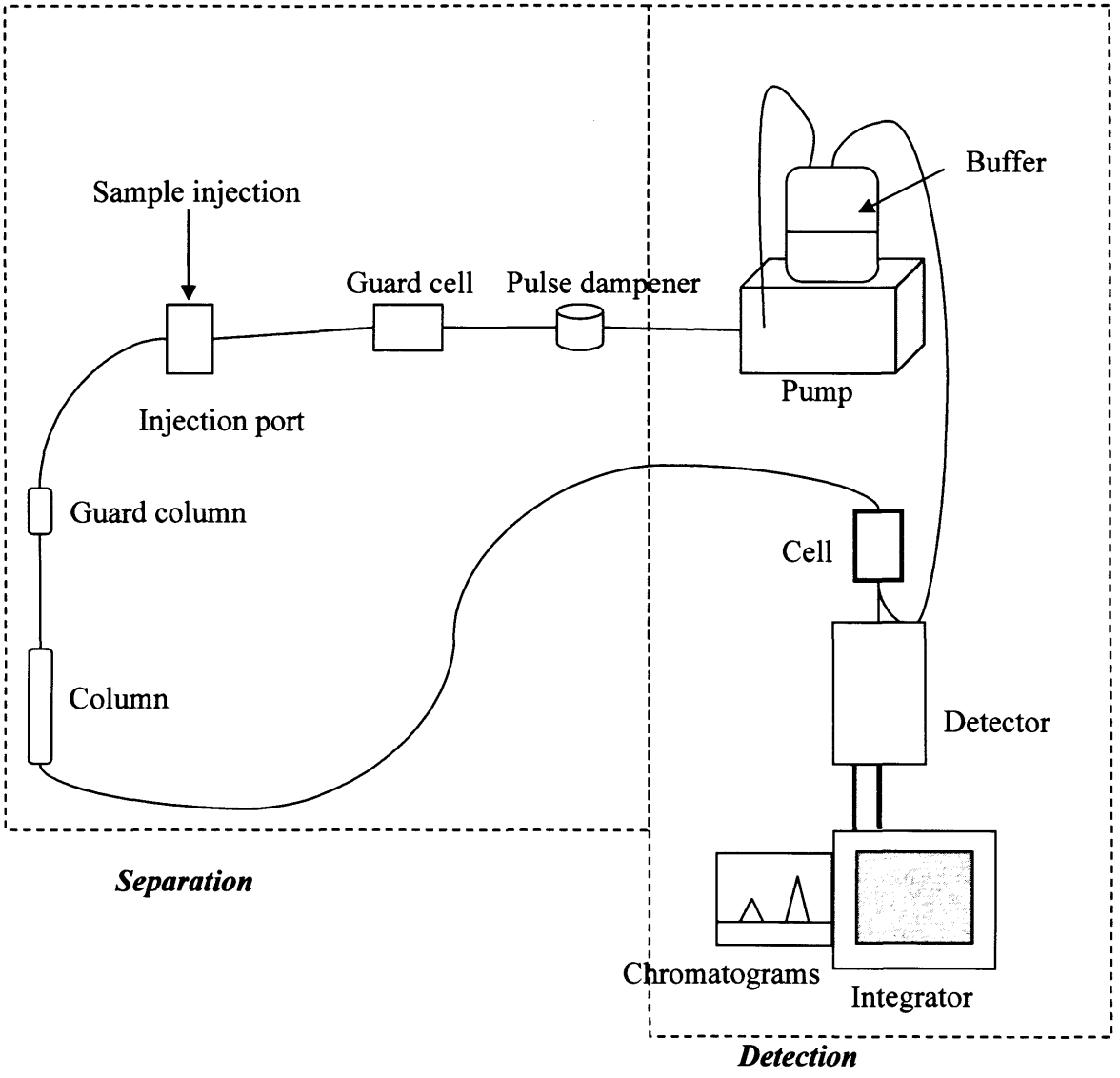


FIGURE 1.4: Schematic diagram of the HPLC / ECD used in these studies.

1.4.2 Control of noradrenaline synthesis

A number of possibilities may explain the differences in basal NA efflux between NK1+/+ and NK1-/- mice found with the pilot study. One may be that there is more NA available for release in NK1-/- mice, as a result of more NA being synthesised.

The biosynthetic pathway leading to the production of NA (Figure 1.5) begins with the amino acid tyrosine (Carson & Robertson, 2002). The initial and rate-limiting step in the production of NA is the hydroxylation of tyrosine by tyrosine hydroxylase (TH) to

L-dihydroxyphenylalanine (DOPA). Mutation of TH in TH-/- mice is lethal (Kobayashi *et al.*, 1995), demonstrating the crucial role played by this enzyme. As TH is the rate-limiting enzyme responsible for conversion of the amino acid tyrosine to L-DOPA, this enzyme was the initial target of preliminary investigations. However, TH also controls the synthesis of NA, dopamine (DA) and adrenaline, so investigations also focused on dopamine-β-hydroxylase (DβH), the vesicle-bound enzyme which converts DA to NA.

DOPA synthesized by TH is decarboxylated by L-aromatic amino acid decarboxylase to DA. In noradrenergic neurones DA is then converted to NA via DβH (Figure 1.5). Therefore, DβH would seem a more selective marker for noradrenergic neurones.

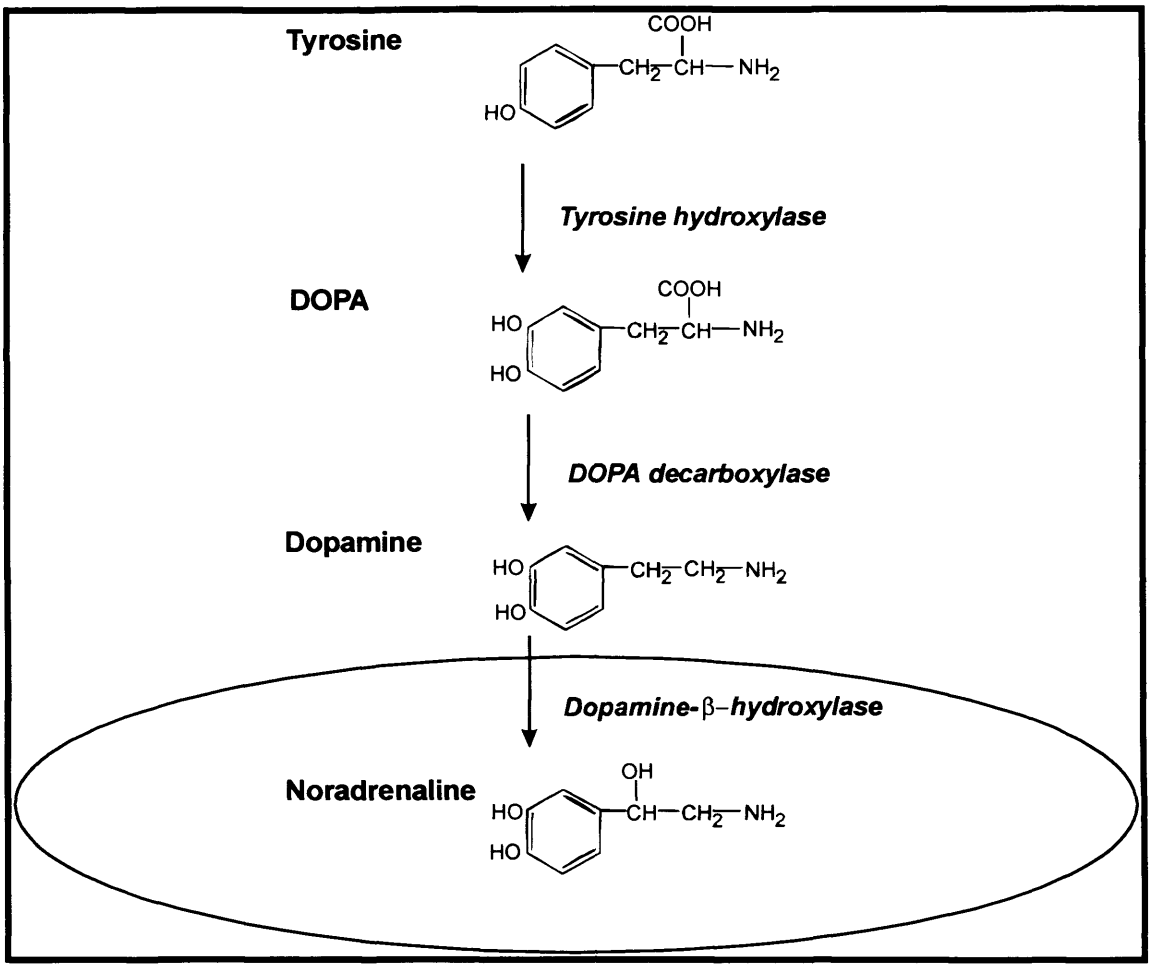


FIGURE 1.5: The synthetic pathway of noradrenaline. The hydroxylation of the amino acid, tyrosine, which forms dihydroxyphenylalanine (DOPA) is the rate-limiting step. Conversion of dopamine to noradrenaline is effected by the vesicular enzyme, dopamine-β-hydroxylase (DβH) after uptake of dopamine into vesicles from the cell cytosol (Taken from Stanford 2001b)

1.4.3 Control of noradrenaline reuptake

The noradrenaline transporter (NAT) is a Na^+ / Cl^--dependent 12-transmembrane-spanning protein residing on pre-synaptic noradrenergic nerve terminals (Carson & Robertson, 2002). The majority of NA is removed from the synaptic cleft via the NAT (Eisenhofer *et al.*, 1992). The importance of the NAT in maintaining neuronal noradrenergic content is demonstrated by the fact that, although there is an increase in the activity of TH in NAT-/- mice, overall there is a 55-70 % reduction in tissue concentration of NA in the prefrontal cortex, hippocampus and

cerebellum (Xu *et al.*, 2000). Furthermore, using microdialysis there was a 60 % reduction in efflux and a 6-fold reduction in NA clearance, resulting in a 2-fold elevation of extracellular NA (Xu *et al.*, 2000). As the NAT regulates the magnitude and duration of NA signalling, by terminating neurotransmission via cellular transport (Blakely *et al.*, 1994), it may also represent an important target underlying the difference in basal NA efflux between NK1-/- and NK1+/+ mice. It was thought possible that the NAT may be functioning less efficiently in NK1-/- mice. Therefore, studies in this thesis, focused on investigating the amount of protein of the NAT in the frontal cortex, hippocampus and LC of NK1+/+ and NK1-/- mice. Desipramine (DMI) is a commonly used antidepressant exerting its actions through inhibition of the NAT. Microdialysis studies, therefore, also investigated the effects of local infusion into the frontal cortex of the noradrenaline reuptake inhibitor (NRI), DMI, on NA efflux in NK1-/- and NK1+/+ mice. If the NAT is the underlying cause for the higher basal NA efflux in NK1-/- mice, it was predicted that DMI infusion would result in a greater incremental increase in NA efflux in NK1+/+ compared to NK1-/- mice. DMI was chosen over another commonly used NRI, reboxetine, due to the known limitations in publishing results associated with reboxetine.

1.4.4 Control of release

1.4.4.1 α-adrenoceptors

Physiological responses to NA are mediated via adrenergic receptors. Adrenoceptors were first classified into α and β adrenoceptors by (Ahlquist, 1948). Later they were divided into α_1-, α_2-, and β-adrenergic receptor types (Bylund, 1988). Figure 1.6 depicts the history of the classification of α-adrenoceptors (for review see: (Civantos Calzada & Aleixandre de Artinano, 2001).

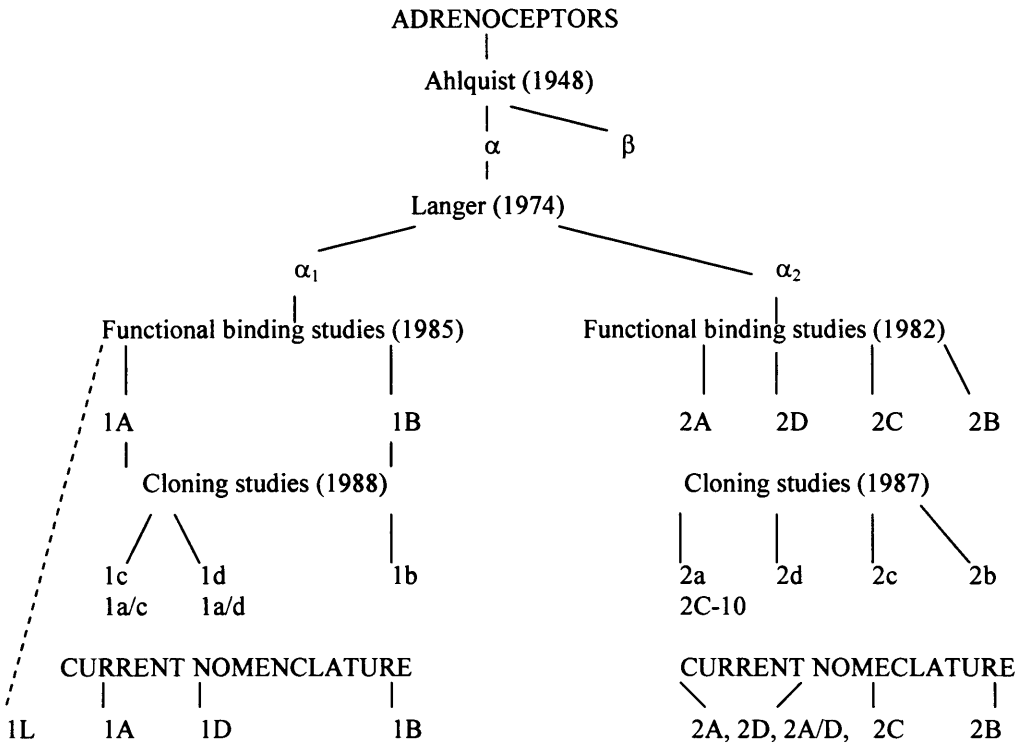


FIGURE 1.6: SCHEMATIC OF THE HISTORY OF THE CLASSIFICATION OF α-ADRENOCEPTORS. ADAPTED FROM (CIVANTOS CALZADA & ALEIXANDRE DE ARTINANO, 2001).

1.4.4.2 α_2-Adrenoceptors

α_2-Adrenoceptors contain seven putative transmembrane α-helices with an extracellular N-terminus and a cytoplasmic C-terminus domain (Bikker *et al.*, 1998).

They are G protein-coupled receptors, responsible for mediating inhibitory effects at the second messenger level. G_o G proteins predominate; these are pertussis toxin-sensitive, rapidly activated and voltage dependent G proteins, in that depolarisation removes the Ca^{2+} channel block. G_i G proteins are pertussis toxin-resistant, slow and voltage insensitive. α_2-Adrenoceptors restrain the synthesis, release and turnover of NA, as well as the firing-rate of noradrenergic neurones. They, therefore, present a prime candidate in elucidating the underlying cause of the higher basal NA efflux in anaesthetised NK1-/- mice.

Activation of α_2-adrenoceptors results in:

- Attenuation of Ca^{2+} rate of entry through voltage-gated Ca^{2+} channels
- Activation of GIRK K^+ channels leading to hyperpolarisation
- Inhibition of adenylate cyclase which results in a decrease in cAMP and consequently a decrease in Ca^{2+} release from internal stores and a decrease in activation of Protein kinase A
- Activation of mitogen-activated protein kinases (MAPK), which may result in the alteration of gene expression and thereby account for some of the neuronal adaptations underlying therapeutic responses to psychotropic drugs as well as neuronal differentiation and survival
- Activation of phospholipase A_2

(for review see: (Brede *et al.*, 2004; Saunders & Limbird, 1999)).

1.4.4.3 Subtypes

Pharmacological interventions and gene targeting strategies from many species has confirmed the existence of three distinct genes for the α_2-adrenoceptor encoding α_{2a}, α_{2b} and α_{2c} (Lomasney *et al.*, 1991; Bylund *et al.*, 1994; Hein & Kobilka, 1995) (for review see: (Philipp *et al.*, 2002; Brede *et al.*, 2004)). The α_{2a} and α_{2d} receptors are species variants of the same receptor (Bylund *et al.*, 1994) with the α_{2a} being found in humans, and α_{2d} being found in rodents and cows. For these studies the $\alpha_{2a/d}$ receptor will be referred to as the α_{2a}-adrenoceptor. Information about their localisation within the brain has been provided through *in situ* hybridisation and immunolabelling techniques.

1.4.4.4 Distribution

Immunolabelling and mRNA expression of all three receptor subtypes is found within the rat and mouse brain with marked regional differences (Scheinin *et al.*, 1994; Wang *et al.*, 1996) (Table 1.4).

Adrenoceptor subtype	mRNA Location in the CNS	Immunolabelling
α_{2a}	Locus coeruleus Brain stem Cerebral cortex Septum Hypothalamus Hippocampus Amygdala Anterior Olfactory nucleus	Diffuse and / or punctate perikaryal expression (Talley *et al.*, 1996). Expression along the plasma membrane of soma in the LC, dendritic trunks and spines in the cortex (Aoki *et al.*, 1994).
α_{2b}	Thalamus Purkinje layer of cerebellum	(Scheinin *et al.*, 1994)*
α_{2c}	Basal ganglia Olfactory tubercle Hippocampus Cerebral cortex	LC (Rosin *et al.*, 1996). Immunoreactivity is found mainly in the neuronal perikarya, with some labelling of proximal dendrites (Rosin *et al.*, 1996).

TABLE 1.4: α_2-adrenoceptor subtype immunolabelling in the central nervous system. *mRNA expression using *in situ* hybridisation.

The α_{2a}-adrenoceptor is the most prevalent within the brain. Approximately 90 % of adrenoceptors in the CNS belong to the α_{2a}-adrenoceptor subtype and the remaining 10 % comprises α_{2b}- and α_{2c}-adrenoceptors (Bucheler *et al.*, 2002). In rats and monkeys there appears to be no inter-species difference in the immunolabelling of the α_{2a}-adrenoceptor (Aoki *et al.*, 1994). However, interspecies differences do exist between the rat and human hippocampal binding of α_2-adrenoceptors (Zilles *et al.*, 1993). Higher α_2-adrenoceptor binding is found in the dentate gyrus and CA3 area of the human hippocampus compared to the rat brain (Zilles *et al.*, 1993).

α_{2a}-Adrenoceptor binding in the human hippocampus (1200 fmol / mg protein) far exceeds that in the rat brain (600 fmol / mg protein).

Localisation of mRNA in the mouse brain follows a similar pattern to the rat; α_{2a} adrenergic mRNA is highly expressed in layer VI of the cortex and the LC. α_{2b}-Adrenoceptors predominate in the thalamus and in the Purkinje layer of the cerebellum and α_{2c} mRNA in the putamen-caudate. Strong expression of both α_{2a} and α_{2c} mRNA is found in the amygdaloid complex, hypothalamus, olfactory system and the hippocampal formation (Wang *et al.*, 1996).

1.4.4.5 Functions

Localisation of the α_2-adrenoceptors, using *in situ* hybridisation techniques and immunolabelling, has not been able to link particular subtypes to physiological functions. Furthermore, lack of subtype-selective agonists and antagonists has made it difficult to assign a specific physiological function to α_2-adrenoceptor subtypes *in vivo*. Advances in elucidating the physiological functions of α_2-adrenoceptor subtypes has been provided by development of mice lacking, over-expressing or bearing a mutation in particular subtypes (MacDonald *et al.*, 1997).

The predominant α_{2a}-adrenoceptor subtype in the brain is the main regulator of pre-synaptic autoinhibition of NA release in the CNS (Trendelenburg *et al.*, 1999; Trendelenburg *et al.*, 2001): that is, they control the release of NA from the neurone itself. The location of the α_{2a}-adrenoceptor in the LC supports its role as a pre-synaptic autoreceptor. These somatodendritic α_{2a}-adrenoceptors (activated via recurrent collaterals) control the firing-rate of LC neurones and NA release from neuronal terminals (Cedarbaum & Aghajanian, 1976; Cedarbaum & Aghajanian, 1978; Aghajanian *et al.*, 1977; Norenberg *et al.*, 1997; Callado & Stamford, 1999; Owesson *et al.*, 2003). α_{2a}-Adrenoceptors confer a high degree of tonic activity onto noradrenergic neurones by mediating feedback inhibition of NA release, ensuring transmitter stores are conserved and preventing excessive stimulation of post-synaptic cells.

Studies using α_{2a}-adrenoceptor knockout mice have confirmed that the α_{2a}-adrenoceptor subtype mediates pre-synaptic and post-synaptic effects of α_2-agonists such as clonidine. These effects include hypotension, sedation, analgesia, hypothermia and also an anaesthetic-sparing effect, likely mediated by the LC (Scheinin & Schwinn, 1992; Guyenet *et al.*, 1994; Lakhlani *et al.*, 1997; Arnsten, 1998; Makaritsis *et al.*, 1999) as well as suggesting that NA release in the medial prefrontal cortex is mainly regulated by α_{2a}-adrenoceptors (Ihalainen & Tanila, 2002). That NA release in the medial prefrontal cortex is mainly regulated by α_{2a}-adrenoceptors confirms pharmacological reports that NA release in terminal areas is tonically modulated by somatodendritic α_{2a}-adrenoceptors located on LC neurones (Mateo *et al.*, 1998; Mateo & Meana, 1999; Fernandez-Pastor & Meana, 2002).

Although α_{2a}-adrenoceptor knock out mice, do not demonstrate an increase in basal NA efflux in the medial prefrontal cortex (Ihalainen & Tanila, 2002), higher plasma NA has been found in α_{2a}-adrenoceptor knockout mice (Makaritsis *et al.*, 1999), demonstrating heightened sympathetic activity in α_{2a}-adrenoceptor knockout mice. However, in α_{2a}-adrenoceptor knockout mice α_2-adrenoceptor agonist induced hypothermia and locomotor inhibition using dexmedetomidine is not totally abolished, suggesting a contribution from α_{2c}-adrenoceptors (Lahdesmaki *et al.*, 2003).

Knockout mice have confirmed that α_{2a} and α_{2c} adrenoceptors exist together pre-synaptically and, although α_{2a}-adrenoceptors play the major role in inhibition of NA release, the α_{2c}-adrenoceptor does contribute in the sympathetic nervous system (Hein *et al.*, 1999) and CNS (Bucheler *et al.*, 2002). α_{2a} And α_{2c}-adrenoceptors can be distinguished functionally in that α_{2a}-adrenoceptors inhibit transmitter release faster and at higher action potential frequencies than α_{2c}-adrenoceptors, which inhibit NA release at lower frequencies (low NA concentrations) (Hein *et al.*, 1999). This correlates with NA possessing a higher potency for α_{2c} than for α_{2a}-adrenoceptors (Hein *et al.*, 1999). Furthermore, the α_{2c}-adrenoceptor subtype has a mainly intracellular localisation (Rosin *et al.*, 1996). Ablation of either α_{2a} or α_{2c} does not result in an upregulation of the other subtype (Bucheler *et al.*, 2002). α_{2a} and α_{2c}-adrenoceptors, therefore, seem to compliment each other in the integration of CNS function and behaviour. Moreover, these receptors play an integral role in regulating neurotransmitter release.

It is, therefore, possible that the higher basal NA efflux observed in NK1-/- mice could be attributed to a decrease in either the density or function of these autoregulatory α_2-adrenoceptors. With this in mind, these studies analysed the localisation and density of α_2-adrenoceptors using immunohistochemistry, Western blot protein analysis and autoradiography (see Sections 2.8-2.10 for a more detailed description of these techniques). As α_2-adrenoceptors are G protein-coupled receptors, the first step in their second messenger signalling pathway (the hydrolysis of GTP to GDP) can be quantified using [^{35}S]GTPγS (see Section 2.11 for a more detailed description of this technique). Agonist-stimulated GTPγS binding was, therefore, used to provide an indication of the functional status of α_2-adrenoceptors in NK1+/+ and NK1-/- mice.

1.4.5 Behavioural response to novelty

For a more detailed description of the LDEB see Section 2.7 and Chapter 5. The light / dark exploration box (LDEB) uses the non-noxious stressors, novelty / light, to elicit behavioural changes that may be attributed to differences in the animals' noradrenergic system. Previous studies in this lab have demonstrated a difference in the behaviour of NK1+/+ and NK1-/- in response to the LDEB (Herpfer *et al.*, 2005). We have further demonstrated that administration of the α_2-adrenoceptor antagonists, atipamezole (Fisher *et al.*, 2003) or yohimbine (Stewart *et al.*, 2002), modify certain behavioural responses in the LDEB only in NK1+/+ mice. Both of these α_2-adrenoceptor antagonists decrease *time to return to the light zone* after first exit so that NK1+/+ mice are indistinguishable from their NK1-/- counterparts treated with either vehicle or the equivalent α_2-adrenoceptor antagonist. Supporting the contention that the noradrenergic system is different between NK1+/+ and NK1-/- mice, perhaps due to a difference in the autoregulatory α_2-adrenoceptor.

The LDEB has the additional benefit in that it can be combined with *in vivo* microdialysis. Previous *in vivo* microdialysis experiments carried out in this laboratory have demonstrated that non-noxious behavioural stimuli e.g. the novel environment, results in an increase in NA efflux (Dalley & Stanford, 1995; McQuade *et al.*, 1999; McQuade & Stanford, 2001) which is strain dependent (McQuade & Stanford, 2001). Conditioning cues, e.g. a tone to indicate transfer to the novel environment also increase NA efflux (McQuade & Stanford, 2000).

1.5 USE OF α_2-ADRENOCEPTOR ANTAGONISTS IN PHYSIOLOGICAL STUDIES

The use of an antagonist in experiments ideally requires a drug that has high specificity for the receptor under investigation (Clarke & Harris, 2002). Studies using α_2-adrenoceptor antagonists to elucidate physiological functions of α_2-adrenoceptors have generally been hampered by a lack of specificity and subtype selectivity for the α_2-adrenoceptor. Yohimbine, or its enantiomer rauwolscine, were the antagonists of choice for probing the physiological function of α_2-adrenoceptors up until the 1980s, as they do not bind to non-adrenergic imidazoline binding sites (Brown *et al.*, 1990; Evans & Haynes, 1994; Uhlen *et al.*, 1995). However, interpretation of results using these compounds is confounded by poor α_2 / α_1 separation (Doxey *et al.*, 1984) and moderate affinity for 5-HT_{1A} receptors (Convents *et al.*, 1989) where they act as agonists (De Vos *et al.*, 1991; Newman-Tancredi *et al.*, 1998).

A number of compounds e.g. the α_2-adrenoceptor antagonist, idazoxan, which are active at α_2-adrenoceptors, also bind to nonadrenergic imidazoline binding sites (Lehmann *et al.*, 1989). These sites have a different neuroanatomical distribution from the α_2-adrenoceptor and therefore, mediate effects independent of actions at the α_2-adrenoceptor for example inhibition of the enzymic activity of monoamine oxidase activity, (for review see: (Holt, 2003)). RX821002 2-[2-(2-methoxy-1,4-benzodioxanyl)]imidazolinehydrochloride, the methoxy derivative of idazoxan, has no appreciable affinity for non-noradrenergic imidazoline binding sites (K_i >10 000 nM; (Langin *et al.*, 1990b; Langin *et al.*, 1990a) and was, therefore, chosen for the studies described in this thesis. The α_2 / α_1 selectivity ratio of RX821002 is 316, whereas that of idazoxan is 151 (Stillings *et al.*, 1985; Welbourn *et al.*, 1986). The rat annococcygeus muscle, is an assay for α_1-adrenoceptor activity, where, RX821002 shows a low affinity (pA_2 4.0) for the α_1-adrenoceptor.

RX821002 was initially believed to bind preferentially to α_{2a}-adrenoceptors as it is not displaced by prazosin, which has moderate affinity for α_{2b} and α_{2c} receptors (Langin *et al.*, 1989). However, binding studies have since revealed that RX821002 binds with high affinity to all α_2-adrenoceptors (O'Rourke *et al.*, 1994) and is therefore, routinely used to investigate total α_2-adrenoceptor binding. The high affinity of RX821002 for

α_2-adrenoceptors is shown by radioligand binding studies with [^{3}H]RX821002 (Devedjian *et al.*, 1994). These studies demonstrate K_d values of 0.29-0.68 nM for the α_{2a}-adrenoceptor subtype, 1.05-2.6 nM for the α_{2b}-adrenoceptor subtype and 0.37-0.55 nM for the α_{2c}-adrenoceptor subtype (Uhlen *et al.*, 1998).

RX821002 *in vitro* is a surmountable, silent and highly selective antagonist at α_2-adrenoceptors, blocking the actions of the α_2-adrenoceptor agonist, UK14304, in the rat vas deferens with a pA_2 of 9.4. The affinity of RX821002 for α_2-adrenoceptors is generally in the range (1 to 6 nM (K_d) (Clarke & Harris, 2002). It has also been shown to behave as an inverse agonist at α_2-adrenoceptors in native tissues expressing normal levels of receptors and G proteins (Murrin *et al.*, 2000). Inverse agonists have a higher affinity for their receptors in the presence of high GTP concentrations, the opposite to what is found with agonists, and they are thought to reduce the functional activity of the receptors below the baseline activity observed in the absence of any ligand. RX821002 has a 5-fold higher affinity, 0.9 nM, for α_2-adrenoceptors in the presence of 100 μM GTP, indicating RX821002 has inverse agonist properties at α_2-adrenoceptors in rat brain tissue sections (Murrin *et al.*, 2000).

A drawback of using RX821002 is that it binds with moderate affinity to 5-HT$_{1A}$-receptors (K_d 20-30 nM) (Grijalba *et al.*, 1996; Newman-Tancredi *et al.*, 1998), where it acts as an antagonist (Newman-Tancredi *et al.*, 1998; Ogilvie & Clarke, 1998). As there is colocalisation of these two receptors, the potential for confusion between the actions at 5-HT$_{1A}$ and α_2-adrenoceptors is great and must be considered when interpreting results. However, previous studies have shown that systemic administration of low doses of RX821002 increases extracellular NA, DA but not 5-HT in the frontal cortex (Gobert *et al.*, 1998). However, it should be noted that the apparent lack of RX821002 on extracellular 5-HT, does not necessarily mean that there is no increase in release, as an increase in 5-HT release could be masked by reuptake via the serotonin transporter.

Alternative compounds available to investigate α_2-adrenoceptor function include atipamezole and MK-912. Atipamezole is virtually inactive at 5-HT$_{1A}$ receptors (Newman-Tancredi *et al.*, 1998). No published reports are available on the binding affinity of MK-912 and atipamezole at imidazoline receptors. Atipamezole, however,

possesses an imidazoline like structure, and studies show that it binds to non-adrenergic sites distinct from I_1, I_2 or I_3 in the rat lung and kidney (Sjoholm *et al.*, 1999; Sjoholm *et al.*, 1995). In the rat spinal cord it is also able to displace dexmedetomidine from non-adrenergic sites, which are thought to represent novel imidazoline binding sites (Savola & Savola, 1996).

Therefore, drawbacks are associated with all the available α_2-adrenoceptor antagonists to date. RX821002 was chosen for these studies as there is little doubt that it does not bind to imidazoline receptors, it is commercially available as a tritiated compound, and has been characterised in a number of assays.

1.6 PROJECT OBJECTIVES

These studies were, therefore, aimed at investigating whether differences exist between NK1-/- and NK1+/+ mice in respect of the control of synthesis, release or reuptake of NA in the frontal cortex and LC, which could explain the difference in basal efflux observed in NK1-/- and NK1+/+ mice.

1.6.1 In vivo microdialysis of cortical noradrenaline efflux in NK1+/+ and NK1-/- mice

- Confirm the preliminary findings that anaesthetised NK1-/- mice possess higher basal efflux of NA in the frontal cortex when compared with their wildtype counterparts (Fisher *et al.*, 2003; Stewart *et al.*, 2004; Herpfer *et al.*, 2005). This involved *in vivo* microdialysis, a technique that monitors changes in NA efflux in the frontal cortex of anaesthetised and freely-moving mice.

- Investigate effect of systemic administration of the α_2-adrenoceptor antagonist RX821002 (0.3, 1.0 and 3.0 mg/kg i.p.) on NA efflux, in the frontal cortex of anaesthetised and freely-moving NK1-/- and NK1+/+ mice.

1.6.2 *Behavioural response to novelty in NK1+/+ and NK1-/- mice*

- Determine genotype dependent behavioural differences in response to the non-noxious stressor, novelty, using the LDEB.

- Ascertain whether any genotype dependent differences in behaviour can be attributed to differences in the noradrenergic system between NK1+/+ and NK1-/- mice. This was achieved by analysis of behavioural parameters after systemic administration of the α_2-adrenoceptor antagonist RX821002 (0.3 mg / kg i.p.).

1.6.3 *Immunohistochemical analysis of the α_2-adrenoceptor, noradrenaline transporter, dopamine-β-hydroxylase and tyrosine hydroxylase*

- Preliminary immunohistochemical investigations of the noradrenergic system in NK1-/- and NK1+/+ mice focused on possible differences in the synthesis of NA, by profiling the location and density of both TH (the rate limiting enzyme) and DβH (the enzyme responsible for the conversion of DA to NA).

- Investigate possible differences in the mechanism of control of NA release and re-uptake. Studies, therefore, focused on profiling the location and amount of the α_{2a}-adrenoceptor and the noradrenaline transporter in the frontal cortex and LC of NK1-/- and NK1+/+ mice.

1.6.4 *Receptor density and Western blot protein analysis*

Although immunohistochemistry (IHC) is a useful method for visualising receptors, transporters or enzymes, further quantification of the amount of the NAT and α_2-adrenoceptor using Western blot protein analysis was required.

- Western blot analysis was used to determine the amount of α_2-adrenoceptor and NAT protein in the LC and frontal cortex.

- Receptor density and localisation were also measured using radiolabelled receptor autoradiography with [^{3}H]RX821002.

1.6.5 Functional activity of α_2-adrenoceptors

The increase in NA efflux in NK1-/- mice may be a cause or consequence of a desensitisation of the autoregulatory α_2-adrenoceptor. [^{35}S]GTPγS binding provides a measure of receptor-mediated G-protein activation. Activation of α_2-adrenoceptors results in exchange of GDP for GTP, which is quantified using [^{35}S]GTPγS.

- Investigate the functional status of α_2-adrenoceptors in NK1-/- and NK1+/+ mice, using the non-hydrolysable GTP analogue, [^{35}S]GTPγS.

The studies contained in this thesis, therefore, use a combination of *in vivo* microdialysis, behavioural and molecular biology approaches to investigate possible candidates that may underlie the higher basal NA efflux in halothane-anaesthetised NK1-/- mice. Any changes in these candidates may be a cause or a consequence of the higher basal NA efflux in anaesthetised NK1-/- mice.

CHAPTER TWO

MATERIALS AND METHODS

2 MATERIALS AND METHODS

2.1 INTRODUCTION

The experiments, described in this thesis, combined behavioural, *in vivo* microdialysis and molecular biology approaches to investigate α_2-adrenoceptors in NK1+/+ and NK1-/- mice. This chapter presents the background to the techniques used. Protocols for each experiment are described in the methods section of each chapter.

2.2 MICE

All experiments described in this thesis used mice or the post-mortem tissue from mice. Mice were housed in Biological Services, UCL, London, in cages of 1-5 animals. Environmental conditions were maintained at 21 °C and 50 % humidity, with tap water and food (Harlan Tekland TRM Rat / Mouse Diet; Harlan, Bicester, UK) *ad libitum*. Lights were programmed on a 12:12 cycle (lights on at 08:00 am). All experiments were conducted in accordance with the UK animals (Scientific Procedures) Act, 1986, and had obtained local ethical approval.

2.2.1 *Neurokinin1 receptor knockout mice*

Adult (25-30 g) male NK1-/- and NK1+/+ mice were used. Mice were derived from homologous recombination of C57BL/6 blastocysts implanted with 129Sv stem cells containing targeted disruption of the NK1 receptor gene (De Felipe *et al.*, 1998); Figure 2.1a. In this mouse line, exon 1 of the gene was disrupted by insertion of a cassette at a unique *Stu*I site. The cassette consisted of an internal ribosome entry site (IRES), the *lacZ* coding sequence and a neomycin resistance gene expressed from its own promoter. These mice were then crossed onto an out bred MF1 background strain (Harlan; (Migaud *et al.*, 1998). Mice were bred from homozygous breeding pairs. Weaning occurred at approximately three weeks of age.

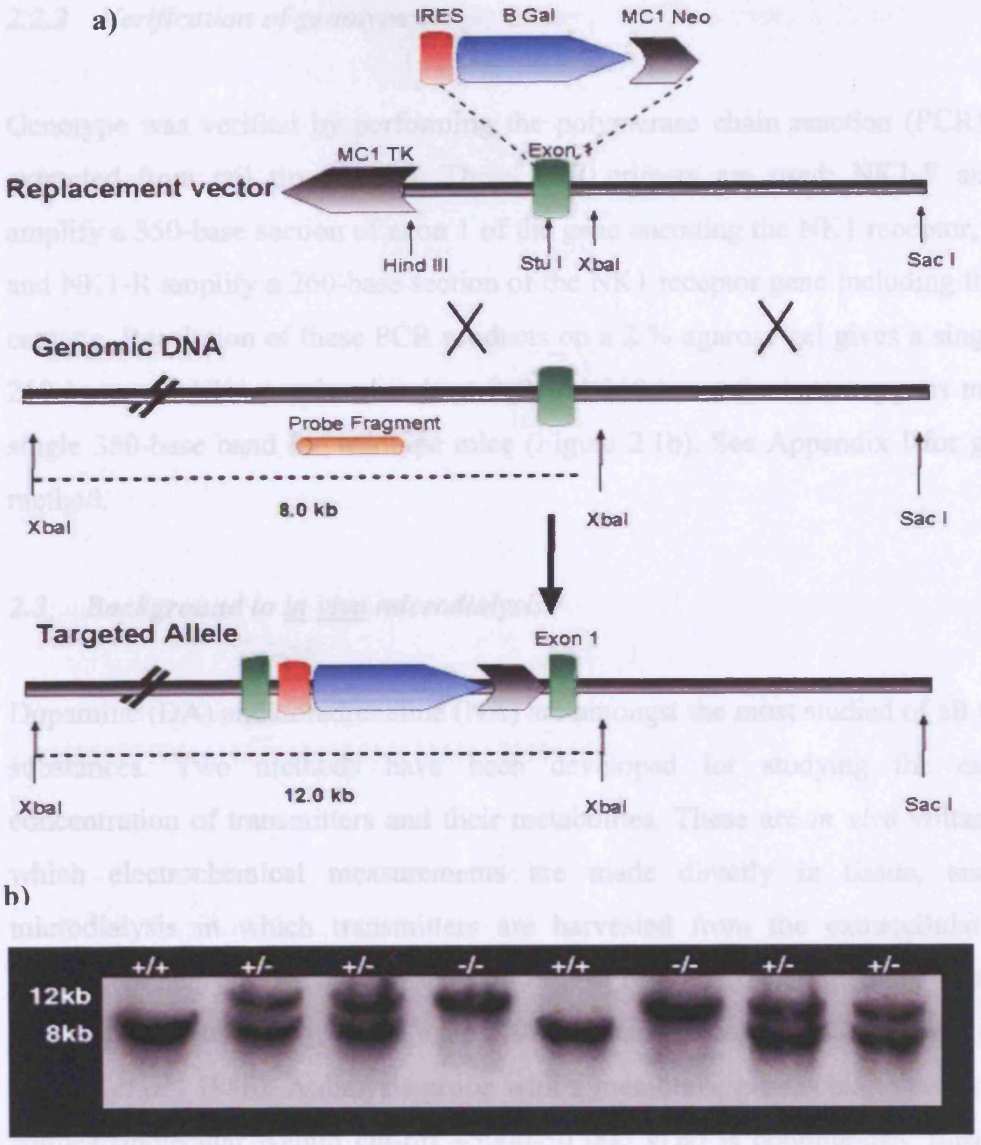


FIGURE 2.1: Disruption of the NK1 receptor, via homologous recombination. a) Schematic of the region of the NK1+/+ locus containing exon 1, the targeting (replacement) vector and predicted structure of the targeted NK1 receptor gene following homologous recombination. b) Analysis of the offspring from a heterozygote NK1+/- intercross using Xba1 digestions of tail DNA and hybridisation with the external 5' probe fragment (orange) and Southern blot analysis. Homozygous (12 kb), heterozygous (12 kb and 8 kb) and NK1+/+ (8kb) generated at a ratio of 1:2:1. BGal: lacZ coding region; IRES: internal ribosome entry site; MC1: MC1 promoter; Neo: neomycin resistance gene: TK: Herpes simplex virus thymidine kinase gene. Restriction sites indicated by small arrows. (Taken from Gadd, 2003; (De Felipe *et al.*, 1998)).

2.2.2 *Verification of genotype*

Genotype was verified by performing the polymerase chain reaction (PCR) on DNA extracted from tail tip samples. Three PCR primers are used: NK1-F and NK1-R amplify a 350-base section of exon 1 of the gene encoding the NK1 receptor, and NeoF and NK1-R amplify a 260-base section of the NK1 receptor gene including the inserted cassette. Resolution of these PCR products on a 2 % agarose gel gives a single band at 260 bases for NK1-/- mice, bands at 260 and 350 bases for heterozygous mice, and a single 350-base band for wildtype mice (Figure 2.1b). See Appendix I for genotyping method.

2.3 *Background to in vivo microdialysis:*

Dopamine (DA) and noradrenaline (NA) are amongst the most studied of all transmitter substances. Two methods have been developed for studying the extracellular concentration of transmitters and their metabolites. These are *in vivo* voltammetry, in which electrochemical measurements are made directly in tissue, and *in vivo* microdialysis in which transmitters are harvested from the extracellular fluid for subsequent analysis. Microdialysis is an *in vivo* brain perfusion method. The technique was originally started by (Sarna *et al.*, 1984; Hutson *et al.*, 1984a; Hutson *et al.*, 1984b; Hutson *et al.*, 1986). A dialysis probe with a membrane permeable to water and small solutes (molecular weight cut-off ~5000-50 000 kDa) is continuously flushed with a solution devoid of the substance of interest. A concentration gradient is created causing diffusion of solutes from the interstitial space across the dialysis membrane and into the probe (Benveniste & Huttemeier, 1990). Three separate water filled compartments make up the brain: intracellular, interstitial (extracellular) and the vascular space (Chaurasia, 1999). The dialysis probe samples from the extracellular fluid (ECF) (Benveniste & Huttemeier, 1990), thereby following changes of the content of neurotransmitter(s) in the ECF compartment of the CNS. Brain dialysis in freely-moving rats has provided major contributions to understanding the mechanism of action of centrally acting drugs on specific neurotransmitters. Furthermore, this information can be paralleled with behavioural changes and with extracellular concentrations of the active drug in drug challenge tests (Chiara, 1990).

One advantage of using microdialysis sampling is that freely-moving animals can be used with minimal tissue damage. Secondly, it is feasible to monitor continuously, changes of neurotransmitters over time, within the extracellular fluid, at more than one probe implantation site (Stenken, 1999). Microdialysis sampling of the tissue takes place generally before, during and after the drug treatment, thus each animal serves as its own control throughout the experiment. This technique, therefore, significantly reduces the number of animals required to complete an experiment. There is also no further risk of enzymatic degradation, once the analyte diffuses into the microdialysate, as the solute is separated from enzymes and other macromolecules, which may cause degradation.

A general problem in microdialysis experiments is that the extremely low concentration of monoamine neurotransmitters in the extracellular fluid requires an analytical detection limit in the low picomol range. Improvements in chromatographic techniques have allowed limits of detection to approach the low femtomol range (Mefford, 1985). Microdialysis, however, is an invasive technique, although not as destructive as the push-pull cannula, developed by Gaddum in 1961 (Gaddum, 1961). This causes extensive tissue damage. Also the perfusion medium is in direct contact with the brain and requires high flow rates (typically 150 µl / min). Insertion of the dialysis probe (several orders of magnitude larger than the structure under study) does cause some tissue damage by breaking blood vessels and disrupting cells (Morgan *et al.*, 1996). The influence of post-probe implantation time is also striking. In the hippocampus 2 h after implantation, marked changes in glucose metabolism and blood-flow are detected in the implanted area (Benveniste *et al.*, 1987). Spreading depression and depolarization in intact neurones also takes place probably as a result of massive K^+ release from damaged elements (Benveniste *et al.*, 1989). 24 h post probe implantation these changes are believed to be resolved (Benveniste *et al.*, 1987).

The key elements of the microdialysis technique are the dialysis probe, connective tubing, perfusion medium and perfusion pump, and sample (dialysate) collection device (Chaurasia, 1999) (Figure 2.2).

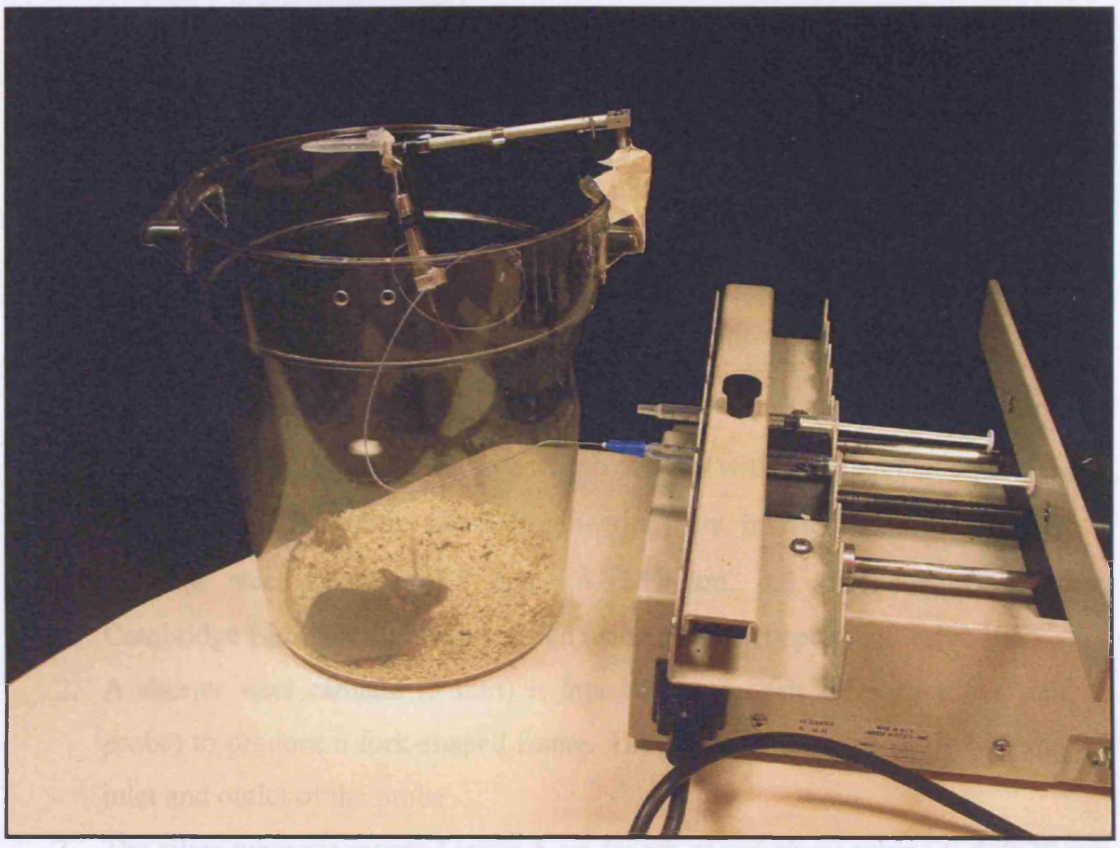


FIGURE 2.2: Photograph of the key elements of the microdialysis technique used in these studies. The probe is perfused using a perfusion pump, with a modified Ringer's solution in a syringe. Samples are collected every 20 mins for analysis using HPLC / ECD.

2.3.1 Probe design and perfusion solution:

Four main types of microdialysis probes are used: a) linear, b) loop, c) side-by-side and, d) concentric (Chaurasia, 1999).

The dialysis membranes of linear probes are formed as cylinders, connected with in- and outlet tubes in serial arrangement. The in- and outlet tubes of concentric, loop and side-by-side designs, are positioned in parallel requiring only a single point of entry. For the present studies the dialysis probe was a single cannula probe and used a side-by-side design with the in- and outlet tubes in parallel. Microdialysis probes in this thesis were made in-house.

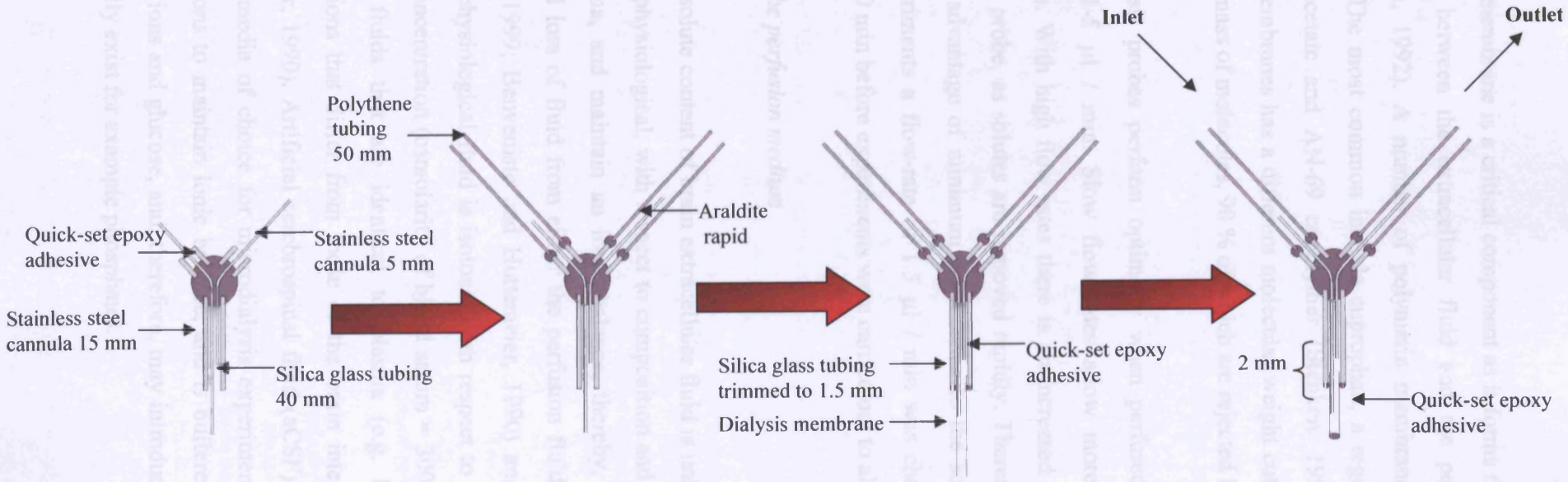
2.3.1.1 Preparation of single cannula microdialysis probe:

The essential materials for constructing a microdialysis probe are:

- Small-diameter semi-permeable dialysis tubing
- Stainless steel tubing
- Water-resistant, all purpose epoxy resin
- Silica capillary tubing

1. Two silica capillary tubes cut to a length of 4 cm with a scalpel blade (i.d. 75 μm, o.d. 150 μm, Scientific Glass Engineering) are inserted side by side into a stainless steel cannula (1.5 cm; i.d. 380 μm, o.d. 500 μm, Goodfellow Cambridge Limited), both are secured using quick-set epoxy adhesive (RS).
2. A shorter steel cannula (5 mm) is attached over each silica tube (top end of probe) to produce a fork-shaped frame. The silica tubing eventually become the inlet and outlet of the probe
3. The silica tubing is inserted into a 5 cm length of polythene tubing (i.d. 0.28 mm, o.d. 0.61 mm, Portex). The polythene tubing is then secured to the stainless steel cannula using quick-set epoxy adhesive (Araldite).
4. The dialysis membrane is carefully passed over the silica inlet tube at the bottom of the probe, which has been trimmed to 1 mm. The membrane is trimmed 1 mm past the silica tubing and sealed with quick-set epoxy adhesive (RS) to give an active window of 1.5 mm.
5. The dialysis membrane is then trimmed 1 mm from the end of the silica glass tubing with a scalpel blade and sealed with quick-set epoxy adhesive (RS). Probes are then stored in an airtight container until use (Figure 2.3).

FIGURE 2.3: Schematic of the step-by-step process of probe preparation used in these microdialysis studies.



The probe membrane is a critical component as it forms the interface for the transfer of substances between the extracellular fluid and the perfusion medium (Sharp and Zetterström, 1992). A number of polymeric membranes are used in microdialysis sampling. The most common include cuprophan, a regenerated cellulose membrane, cellulose acetate and AN-69 copolymer (Stenken, 1999). Each of these different polymer membranes has a different molecular weight cut-off. Cut-off is defined as the molecular mass of molecules, 90 % of which are rejected by the membrane.

Microdialysis probes perform optimally when perfused at constant, low flow-rates generally 1-5 µl / min. Slow flow rates allow more time for analytes to reach equilibrium. With high flow rates there is an increased risk of creating a solute void around the probe, as solutes are removed rapidly. Therefore, slow flow rates have the additional advantage of minimum disturbance to the solute system under study. For these experiments a flow-rate of 1.5 µl / min was chosen. The dialysis probe was perfused 60 min before experiments were carried out to allow stabilisation of the probe.

2.3.1.2 The perfusion medium

The exact solute content of brain extracellular fluid is unknown. The perfusion medium should be physiological, with respect to composition and pH, so that they are isosmotic with plasma, and maintain an ionic balance, thereby, minimising osmotic pressure effects and loss of fluid from either the perfusion fluid or the tissue interstitial fluid (Stenken, 1999; Benveniste and Huttemeier, 1990) and have a stable physiological status. A physiological fluid is isotonic with respect to blood serum, i.e. of the same osmotic concentration (osmolarity of blood serum = 300 mMol) and also composition. Perfusion fluids that are identical to plasma (e.g. Ringer's solutions) have ion concentrations that differ from those of the brain interstitial space (Benveniste and Huttemeier, 1990). Artificial cerebrospinal fluid (aCSF) and Ringer's solution are the perfusion media of choice for microdialysis experiments. Ringer's solution contains essential ions to maintain ionic balance, and is buffered by the CSF. aCSF contains additional ions and glucose, and, therefore, may introduce ions into the brain which do not normally exist for example phosphates.

Composition of Ringers:

- KCl 4 mM
- NaCl 145 mM
- $CaCl_2$ 1.3 mM

Dissolved in distilled water pH 7

2.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION FOR ANALYSIS OF NORADRENALINE

2.4.1 High Performance Liquid Chromatography

In chromatography, separation is achieved through 'differential rates of migration', meaning that different solutes move through the system at different velocities. Due to the versatility of liquid chromatography, it is the main method of choice employed to measure compounds of interest in microdialysates. The liquid chromatography method not only provides a degree of selectivity necessary for the analysis of complex mixtures, it also enables the microdialysis sampling to determine simultaneously more than one substance (see: (Chaurasia, 1999)). Analysis using High Performance Liquid Chromatography with Electrochemical Detection (HPLC / ECD) is sufficiently rapid to allow perfusates to be analysed continuously as the experiment progresses.

2.4.1.1 Column

The studies in this thesis used reversed-phase ion-pair chromatography which is one of the most widely used separation methods for monoamines.

Reversed phase chromatography consists of a nonpolar stationary phase and a polar mobile phase. Nonpolar compounds partition between the polar solvent and the nonpolar support (Mefford, 1985). Ion-pairing is achieved by addition of an ion-pairing reagent to the aqueous organic eluent e.g. a detergent such as octanesulfonic acid (OSA), as used within these studies. Silica-based stationary phases are the most common in HPLC. The OSA forms a hydrophobic bond with the silica stationary phase. Polar amines then form an ion-pair with the OSA. The rate at which solutes move through the

column is dependent on surface area of the column (related to diameter of the spherical packing material), and the type of hydrophobic layer. The type of column used in HPLC / ECD depends on the solutes to be separated, the primary requirement being the use of polar, primary aqueous solvents containing some ionic species. The columns used in this study were octadecylsilane (ODS) columns: the stationary phase consisted of silica derivatised with octadecyl groups.

2.4.1.2 High Performance Liquid Chromatography mobile phase

The main role of the mobile phase is to determine the retention time of the solute. If solutes elute too quickly, they are not separated from the solvent front. If retention time is too long, the peak widens, peak height is reduced, and sensitivity is decreased. The retention time of the solute can be altered by modifying the mobile phase in the following way:

- An increase in pH of the buffer decreases the retention time of the solute (only if p*K* differs from 7).
- An increase in the concentration of ion-pairing agents e.g. OSA in the buffer increases the retention time of monoamines
- An increase in the amount of organic solvent in the buffer decreases the retention time of monoamines.

(Sharp and Zetterström, 1992)

High Performance Liquid Chromatography mobile phase components:

- 2 mM Octanesulfonic acid (OSA) (98 %, Sigma)
- 100 mM NaH_2PO_4 (99 %, Fischer)
- 0.67 mM EDTA (99.5 % AnalaR)
- 12 % Methanol (99.8 %, Prolabo)

All dissolved in distilled H_2O, pH adjusted to 3.75. Under these conditions NA was separated out from the solvent front with a retention time of around 8-9 min. The mobile phase was degassed by agitation and vacuum filtration using FTK membrane filters (Pall Process Filtration). The buffer was recycled for 3-4 months.

2.4.1.3 Components of High Performance Liquid Chromatography system:

- Shimadzu LC 6A isocratic pump ESA 582 delivering mobile phase at a flow rate of 1.0 ml / min
- Pulse dampener (ESA)
- A 50 µl injection stainless steel loop (Anachem) attached to an injection port: Rheodyne 7125
- A guard column: Aquapore G.C. 7 µm, 4.6 x 30 mm, Brownlee, Perkin Elmer
- A column: Hypersil ODS; 5µm packing; 4.6 x 250 mm: Thermo Hypersil Ltd., U.K.;

2.5 ELECTROCHEMICAL DETECTION SYSTEM, ITS THEORY AND COMPONENTS

The type of analyte as well as sensitivity of the HPLC / ECD system, dictates the choice of detector used. The method of detection chosen for these experiments is electrochemical detection (ECD), which is well established for amino acids, choline / acetylcholine, glucose and amines such as DA, NA and 5-HT (Marsden *et al.*, 1990; Ruban, 1993; Cheng & Kuo, 1995). The detector output is integrated and analyzed using computer-based (Dell) chromatography hardware / software system (TurboChrom). A computer-based system such as this is convenient for data collection, analysis, and storage, although slow changes in system sensitivity and baseline noise are overlooked more easily through human error than if a chart recorder is used (Abercrombie and Finlay, 1991).

2.5.1 Components of the ECD used in these studies

- Guard cell ESA model 5020 +350 mV
- Electrochemical cell: model 5014B consisting of a porous graphite reference electrode set at -280 mV and a porous graphite measuring electrode set at + 180 mV in series. This cell uses coulometric detection, so that 100 % of the analyte is oxidised and recycled in the buffer. This is in contrast to amperometric detection, which uses glassy carbon electrodes. Using amperometric detection the signal from approximately 95 % of the solute is not generated.

- Coulochem detector ESA model 5100 A
- Integrator: TurboChrom package

The electrochemical detection is a reduction-oxidation reaction and takes place at the cell. The negative potential, applied to the first electrode, conditions the mobile phase containing the solutes by donating an electron (reduction). Detection occurs at the second electrode which is set at a positive potential, and accepts electrons from the reduced solute (oxidation). The flow of electrons during the electrochemical reaction gives rise to a current measured by the detector. The magnitude of the current generated is related to the concentration of the solute being reacted. A standard resistance is found through the HPLC / ECD system. According to Ohm's law, therefore, the current is expressed as a peak, measured as (μV) on the chromatogram.

2.5.1.1 Determination of oxidation potential for Electrochemical Detection

The measuring electrode is fixed at a potential which maximises the signal : noise ratio. As mentioned previously, detection takes place in a coulometric cell, which is set at the optimum potential. To determine this optimal potential it was necessary to construct a current / potential calibration curve for NA. This was achieved by injecting 50 μl NA at a concentration of 100 fmol into the HPLC / ECD. The measuring electrode was set at a range of potentials from -20 to +380 mV. The voltammogram obtained is shown below (Figure 2.4). A plateau is observed from + 200 mV to + 380 mV but from + 200 mV to -20 mV the NA peak declines sharply *linearly.* From these results, it was decided that the measuring electrode should be set at +180 mV. The optimal electrode potential should be low enough to prevent the oxidation of too many impurities in the samples, as this could interfere with the NA peak, but high enough to ensure the electroactive species reaching the electrode react.

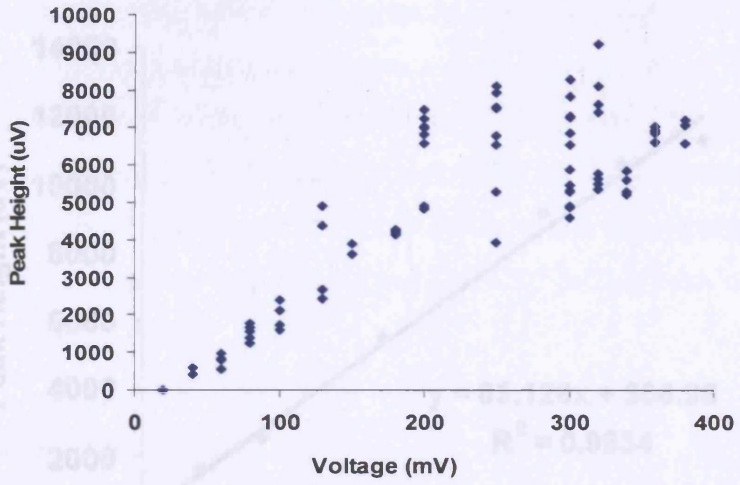


FIGURE 2.4: Voltammogram obtained by oxidation of noradrenaline at electrode potentials ranging from -20 to +380 mV.

2.5.1.2 Calibration and determination of detection limit of the HPLC / ECD system

Regular calibration of the HPLC / ECD system was necessary to ensure accurate measurement of the NA peak on the chromatogram, as well as determination of the detection limit of the system. This was achieved by injecting 50 µl NA at concentrations ranging from 2 to 150 fmol with the electrode set at the optimum potential described above (180 mV) for the ECD. From 150 fmol / 50 µl down to 2 fmol / 50 µl there is a linear relationship between peak height and NA concentration (R^2=0.99) (Figure 2.5). Since the signal at 4 fmol was more than twice that of the basal noise, it was concluded that under these conditions 2-4 fmol was the detection limit for NA.

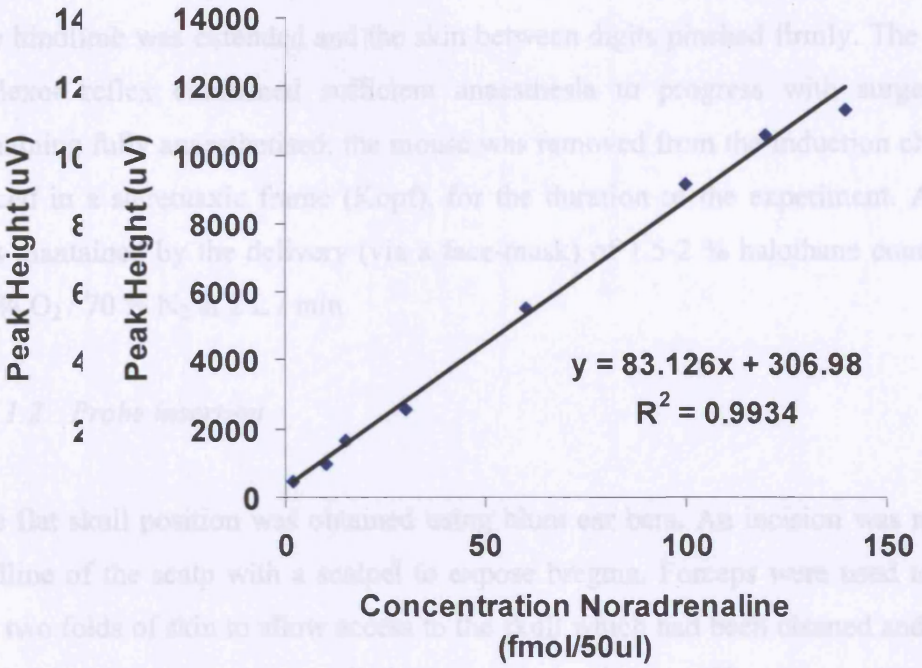


FIGURE 2.5: Calibration curve for the detection of noradrenaline. The height of the peak representing oxidation of noradrenaline was plotted (Y-axis) the concentration of noradrenaline in the sample (X-axis). The height of the peak follows a linear relationship down to 2 fmol noradrenaline.

Calibration of the HPLC / ECD was carried out every day at the start of every experiment.

2.6 IN VIVO MICRODIALYSIS

2.6.1 Surgical procedure

2.6.1.1 Non-Recovery (continuous anaesthesia) in vivo microdialysis

Induction of anaesthesia. An oxygen cylinder fitted with a pinindex regulator and flow meter was connected to a purpose-built vaporiser to provide smooth delivery of inhalational anaesthesia (2.0 % halothane combined with 30 % O_2 / 70 % N_2 at 2 L / min). The halothane was piped into a transparant induction chamber (12.5 cm x 13.5 cm

x 26.5 cm) enabling observation of the animal during induction of anaesthesia. To ensure sufficient depth of anaesthesia the animal's pedal withdrawal reflex was tested. The hindlimb was extended and the skin between digits pinched firmly. The absence of a flexor reflex confirmed sufficient anaesthesia to progress with surgery. Whilst remaining fully anaesthetised, the mouse was removed from the induction chamber and placed in a stereotaxic frame (Kopf), for the duration of the experiment. Anaesthesia was mantained by the delivery (via a face-mask) of 1.5-2 % halothane combined with 30 % O_2 / 70 % N_2 at 2 L / min.

2.6.1.2 Probe insertion

The flat skull position was obtained using blunt ear bars. An incision was made in the midline of the scalp with a scalpel to expose bregma. Forceps were used to withdraw the two folds of skin to allow access to the skull which had been cleaned and dried. The flat skull position was assumed when ventral coordinates of bregma, and two positions ± 1.0 mm lateral to bregma, were within 0.1 mm. Using a trepanning drill burr, a 1mm diameter hole was drilled into the skull where the probe was to be inserted: mm frontal cortex: AP +2.1, ML +1, DV -2 according to the mouse atlas of Paxinos and Watson (2001). The dura was broken with a needle and a probe, primed with modified Ringer's solution (NaCl 145 mM, KCl 4 mM, $CaCl_2$ 1.3 mM), placed at the coordinates given above. The probe was then slowly lowered vertically into its final position: mm frontal cortex: DV –2 mm.

The probe was inserted into the secondary motor (M2) cortical region (Figure 2.6). This brain region was chosen based on its topographical relationship with subcortical regions, as it most closely resembles that of the FR2 region in the rat brain, which we have previously studied (McQuade *et al.*, 1999). In the rat, the FR2 region receives dense noradrenergic input from the LC and vice versa (Sara & Herve-Minvielle, 1995).

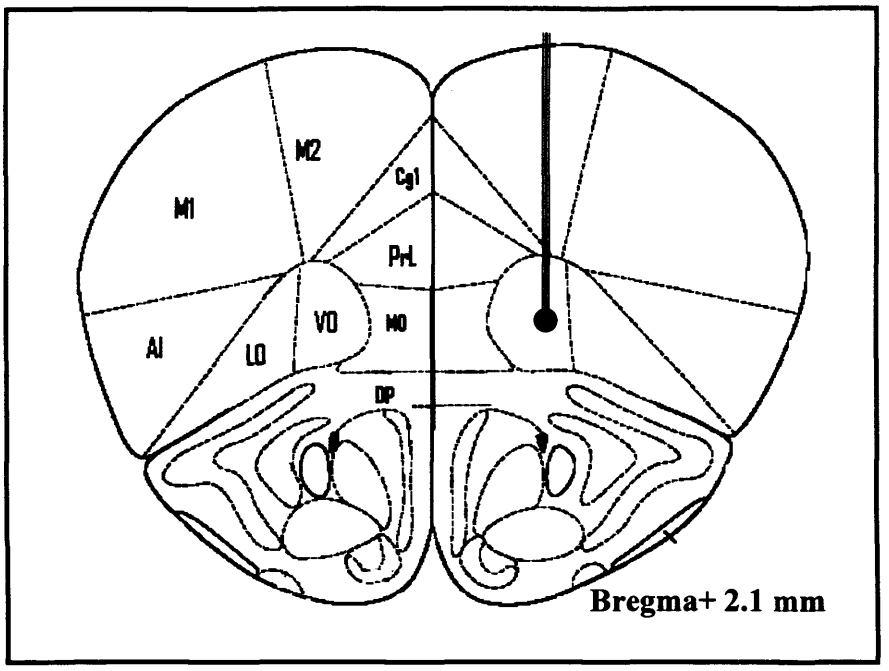


FIGURE 2.6: Coronal schematic of the site of probe insertion in the M2 region of the mouse prefrontal cortex. Not drawn to scale.

After probe implantation, the mice were maintained under anaesthesia for the duration of the experiment. Vital signs were monitored continually to ensure a respiratory rate of 100-190 / min (average: 135 ± 20 / min). Core body temperature was maintained at 38 °C using a warm water-bed. Sterile saline (0.9 %, 0.1 ml) was injected subcutaneously, once per hour, to prevent dehydration. Microdialysis probes were perfused with modified Ringer's solution at a rate of 1.5 µl / min and dialysis samples collected at 20 min intervals. A drug challenge started after a minimum of three consecutive basal samples indicated a stable baseline for spontaneous NA efflux.

2.6.2 Freely-moving in vivo microdialysis

Induction of anaesthesia was as described in Section 2.6.1.1 but using a 95 % O_2 and 5 % CO_2 mix at 250 cc / min.

Maintenance of anaesthesia: Whilst the animal remained fully anaesthetised, it was placed in a stereotaxic frame (Kopf) where anaesthesia was mantained by the delivery

(via a face-mask) of 1.5-2 % halothane combined with 95 % O_2 / 5 % CO_2 at 250 cc / min.

Prior to probe insertion as decribed in section 2.6.1.2 two bone anchor self tapping screws (Bioanalytical Systems), were inserted on either side of the skull, at the level of the parietal bone to anchor the cyanoacrylate gel that would secure the probes after their insertion (Figure 2.7).

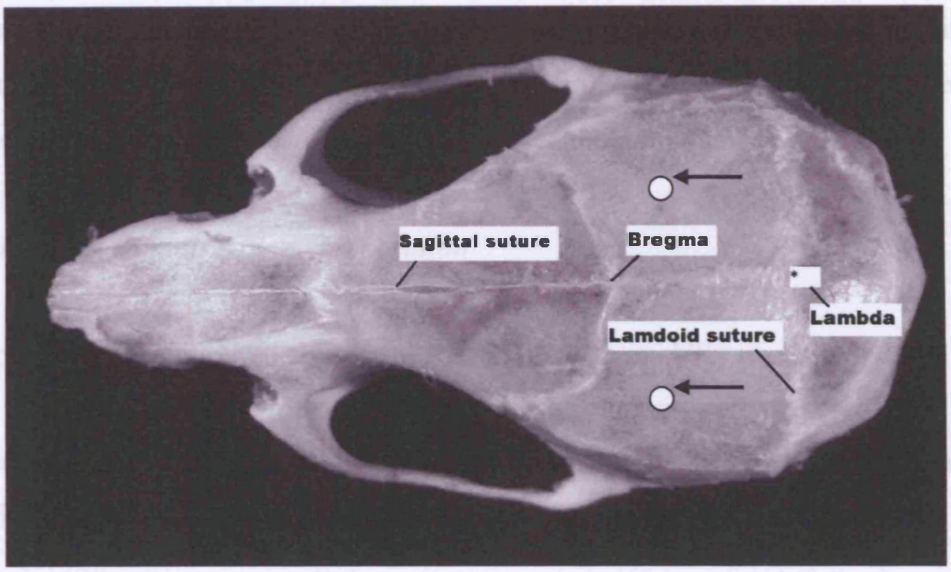


FIGURE 2.7: The dorsal surface of the mouse skull showing placement of the self tapping bone screws for securing the dialysis probe in freely-moving mouse microdialysis indicated by arrows (Taken from Paxinos and Watson, 2001).

The probe was then secured in place using cyanoacrylate gel glue and activator (RS), taking care to avoid the eyes and whiskers of the mouse (Figure 2.8).

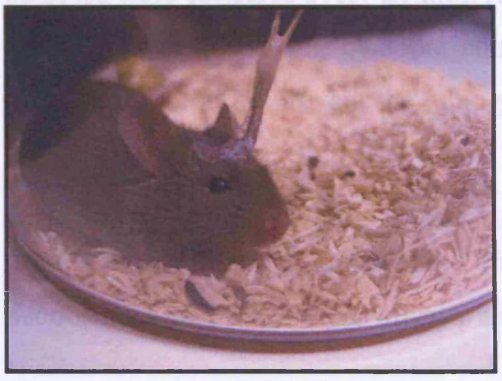


FIGURE 2.8: Photograph of the probe secured in place with cyanoacrylate gel glue.

A collar, made of card, was also attached around the head of the mouse to prevent removal of the probe during the night. The mouse was placed in a plastic cage (Harvard Apparatus; diameter 8 cm height 21 cm), with free access to wet mash. The plastic cage was then placed in an incubator to allow the mouse to recover from anaesthesia. Based on histological, functional, metabolic and blood-flow changes caused by the probe insertion, it appears that the optimal time for commencing microdialysis is 8-48 h after probe implantation (Benveniste and Huttemeier 2003). For the freely-moving mouse dialysis, experiments were, therefore, typically carried out 12-24 h after probe implantation. The inlet and outlet of the probes were connected, through FEP tubing (i.d: 0.12 mm o.d: 0.68 mm; dead volume: 1.2 µl / 10 cm), to a low-torque quartz-lined dual channel swivel (Instech). Dead volume was kept to the minimum.

2.6.2.1 Drug administration / preparation

For i.p. dosing, the required amount of RX821002 (Sigma,UK) was dissolved in 0.9 % saline to give a dosing volume of 10 ml / kg. Fresh solutions were prepared daily. When infused locally into the terminal field, via the dialysis probe, desipramine was diluted in modified Ringer's solution.

2.6.2.2 Collection of dialysates

At 08:00 h on the day after surgery, mice were transferred to the experimental laboratory. The cuff was removed and the inlet of the swivel connected via FEP tubing (i.d: 0.12 mm o.d: 0.68 mm Royem Scientific Ltd.) to a 1 ml disposable syringe (BD Plastipak™) filled with modified Ringer's solution and perfused using a Kd Scientific perfusion pump at 1.5 µl / min. The outlet of the swivel was then connected, using FEP tubing, to long-form, tapered polythene tubes (Hughes and Hughes Ltd.) where the samples were collected every 20 min. A counterbalanced lever arm (Harvard Apparatus) held the swivel (Instech) over the animal container (Harvard Apparatus). The swivel was used in order to prevent the tubing from the inlet and outlet of the probe becoming entangled.

Samples were analysed immediately after collection using HPLC / ECD as described in sections 2.4-5. Probes were always allowed to equilibrate for 60 min before starting

collection of basal samples. Once a stable baseline had been achieved with three successive samples, RX821002 was administered. Depending on experimental design, samples were then collected for periods ranging from 120-240 min. The animal was then killed using a Schedule 1 method (see Section 2.6.3). Brains were then removed from the skull and fixed in 4 % formalin and then stained with cresyl violet for confirmation of probe placement (Figure 2.9).

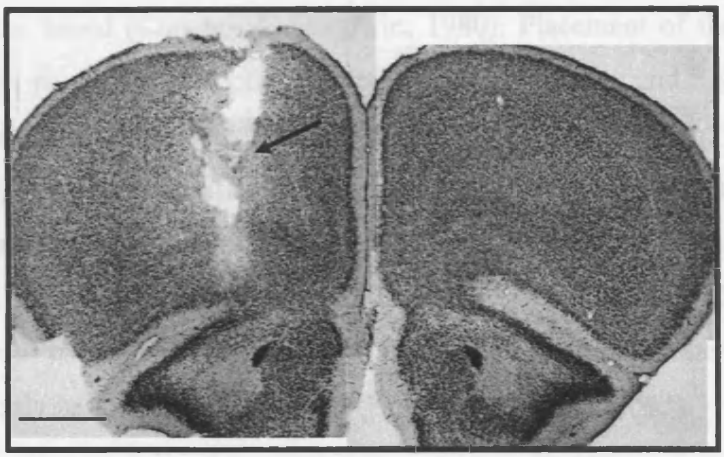


FIGURE 2.9: Confirmation of probe implantation in the secondary motor (M2) region, bregma + 2.1mm. Arrow indicates probe track. Scale bar = 750 μm.

2.6.2.3 Calibration of the HPLC-ECD system

NA standards of 15, 30 and 60 fmol / 50μl were injected daily. The concentration of the test solutes was estimated using a calibration curve of external NA standards.

2.6.3 Schedule 1

After microdialysis experiments, mice were given an overdose of anaesthetic, and a sample of NA efflux collected, to confirm NA had been sampled, as NA efflux increases substantially during hypoxia. Spinal cords were then severed and their brains removed for confirmation of probe placement.

2.7 *BEHAVIOUR*

2.7.1 *Light Dark Exploration Box*

We used a light dark-exploration box (LDEB) based on that first described by (Crawley & Goodwin, 1980) but with a different protocol.

Mice have an innate aversion to the brightly lit environment, and it is on these factors that the LDEB is based (Crawley & Goodwin, 1980). Placement of the animal into a novel brightly lit environment applies the mild stressors novelty and light. The conflict that arises when an animal is exposed to an unfamiliar environment is between the tendency to explore and the initial tendency to avoid the unfamiliar (neophobia). The exploratory behaviour is a reflection of a balance of these tendencies in novel situations (Hascoet *et al.*, 2001; Bourin & Hascoet, 2003). An advantage of the LDEB, is that it is quick and easy to use. No prior training of animals, or food and water deprivation, is necessary and only natural stimuli are used (Bourin & Hascoet, 2003).

Typical dimensions of the compartment are one third for the dark compartment and two thirds for the light compartment. In this model the safe area is the small dark compartment (one third), to which the animals have been allowed to habituate. In the studies performed by Crawley and Goodwin, (1980) no habituation took place. The aversive area is the large illuminated compartment. The fact that inbred mice show substantial differences in their spontaneous behaviour supports the belief that choice of strain, is important (Bourin & Hascoet, 2003).

The LDEB, however, can produce false positive results if a drug increases general activity. It, therefore, seems reasonable to suggest that preliminary screening of locomotor activity (such as open-field or an actimeter test) is advisable before conducting the LDEB, as this would eliminate false positives. We have tested the animals' ambulatory activity and found it to be greater in NK1-/-, than NK1+/+ mice (Herpfer *et al.*, 2005). NK1-/- mice also demonstrate higher locomotor activity in the LDEB (Herpfer *et al.*, 2005).

2.7.1.1 Apparatus (light/dark exploration box)

All behavioural tests were carried out (13:00-17:30) in a LDEB comprising a light compartment serving as the novel 'test zone' (30 cm long x 18.8 cm wide x 24 cm high) and a dark 'neutral zone' (15 cm long x 18.8 cm wide x 24 cm high) (Figure 2.10).

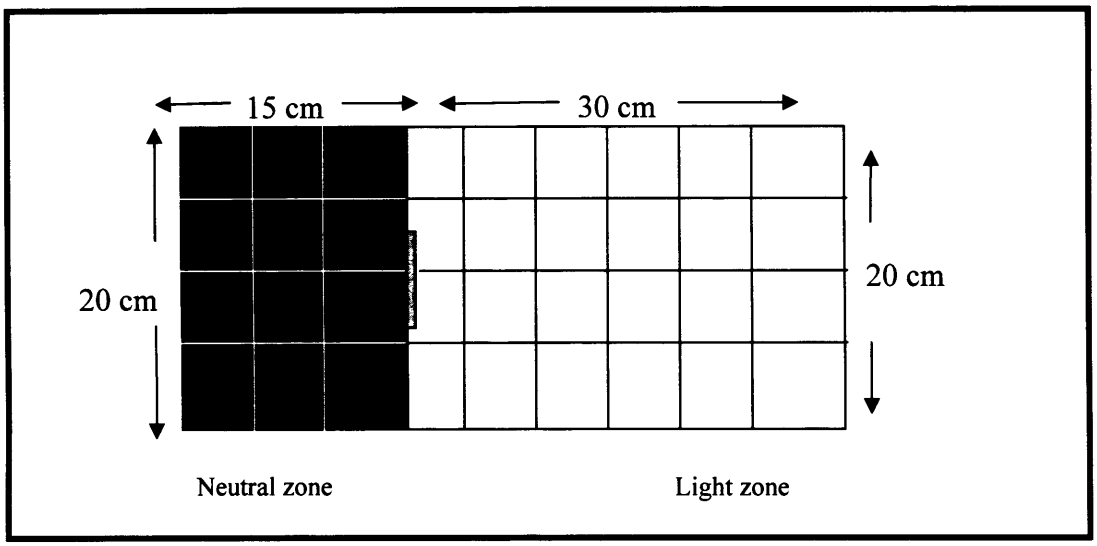


FIGURE 2.10: Schematic of the LDEB used in these experiments. The LDEB comprised of a light novel zone and a dark zone, to which the mice had been allowed to habituate. The two compartments are separated by a guillotine style door, coloured grey in this schematic.

The walls and floor of the 'neutral zone', illuminated at 2 lux, were coloured black. The walls and floor of the 'light zone' illuminated at 10 lux were coloured white. The floor of the light arena was divided into 24 squares (4.5 cm^2) whereas that of the neutral arena was divided into 9 squares (4.5 cm^2). The partition between the neutral and light zones incorporated a white guillotine style door (30 cm x 7 cm), which could be raised to allow the mice to traverse freely between the two compartments.

2.7.1.2 Behavioural scoring

The following behavioural parameters were measured in both the light and neutral arena:

- ***Number of lines crossed by the mouse*** (as an index of locomotor activity), a diagonal crossing of the whole body counted as two lines, a vertical or horizontal line crossing recorded as one.
- ***Number of rears*** (recorded when animal was on hind feet).
- ***Number and duration of grooming episodes***.
- ***Risk assessment behaviour;*** including numer of stretched-attend postures and time spent in flat back approach.

Behavioural parameters measured only in the light zone included:

- ***Latency to leave*** the light arena following forced entry (Active avoidance)
- ***Latency to return*** to the light arena following the first exit (Passive avoidance)
- ***Number of returns*** to the light arena
- ***Total time*** spent in the light arena. The appearance of all four paws was the criterion for re-entry to the light zone, and each 'line-crossing' had to include both the hind paws. Behaviours were scored within 10 minute bins and summary data calculated.

2.7.2 In vivo microdialysis in the light dark exploration box

In vivo microdialysis has previously been used to investigate changes in NA efflux caused by novelty as a non-noxious stressor in Maudsley rats (Dalley & Stanford, 1995; McQuade *et al.*, 1999). The marked genotype-dependent behavioural differences, observed between the NK1+/+ and NK1-/- mice, in response to a novel environment have not been investigated using *in vivo* microdialysis. For this reason, these experiments used *in vivo* microdialysis in the LDEB, to determine whether the noradrenergic response to novelty differs between NK1+/+ and NK1-/- mice.

2.7.2.1 Surgical procedure

As described in section 2.6.2

2.7.2.2 Apparatus

As described in section 2.7

2.7.2.3 Protocol

See Chapter 5.

2.8 IMMUNOHISTOCHEMISTRY

2.8.1 Background to immunohistochemistry

Immunohistochemistry (IHC) is a method of detecting the presence of specific proteins in cells or tissues. The technique consists of a primary antibody binding to a specific antigen of interest, the antibody-antigen complex is bound by a secondary antibody which is generally biotinylated. The secondary biotinylated antibody binds to the antibodies produced by the species in which the primary antibody was raised. Application of avidin bound to the fluorescent label or horse-radish peroxidase results in the formation of a macromolecular complex around the antigen which is visible under the light or fluorescent microscope.

2.8.2 Tissue preparation and fixation

Male NK1+/+ and NK1-/- mice (25-30 g) were deeply anaesthetised with sodium pentobarbital (0.2 ml i.p; Merial) and perfused through the left ventricle of the heart with heparinised saline, followed by fixative containing 4% paraformaldehyde in phosphate buffer (PB) 0.1 M, and distilled water. The brain was removed from each animal and post-fixed in the same solution for 2-3 h and then transferred to a solution containing 30 % sucrose and 0.02 % azide.

Tissue blocks containing the frontal cortex and LC were mounted onto a freezing microtome (SM 2000 R; Leica Microsystems, Milton Keynes, UK) and frozen with dry ice. Serial coronal sections 40 μm thick were collected into 5 % sucrose in 0.1 M PB and stored at 4°C.

2.8.2.1 Primary Antibodies

Table 2.1 lists the primary antibodies used in the experiments contained in this thesis. The general step-by-step process for the IHC used in these studies is detailed in Figure 2.11.

Antigen	**Animal in which raised**	**Monoclonal or polyclonal (M/P)**	**Detection method**	**Optimal dilution**	**Source**
$Alpha_{2a}$	Rabbit	P	TSA	1:50 000	A gift from Pete O'Hara San Francisco
$Alpha_{2a}$	Rabbit	P	DAB FITC	1:1000	Affinity Bioreagents
$Alpha_{2a}$	Goat	P	DAB FITC	1:1000	Santa Cruz
Dopamine-β-hydroxylase (DβH)	Rabbit	P	DAB FITC	1:1000	Research diagnostics
Noradrenaline transporter (NAT)	Rabbit	P	DAB FITC	1:1000	Chemicon
NK1 receptor	Rabbit	P	DAB FITC	1:10 000	
Tyrosine hydroxylase (TH)	Goat	P	DAB FITC	1:5000	Calbiochem

TABLE 2.1: The primary antibodies used in these studies.

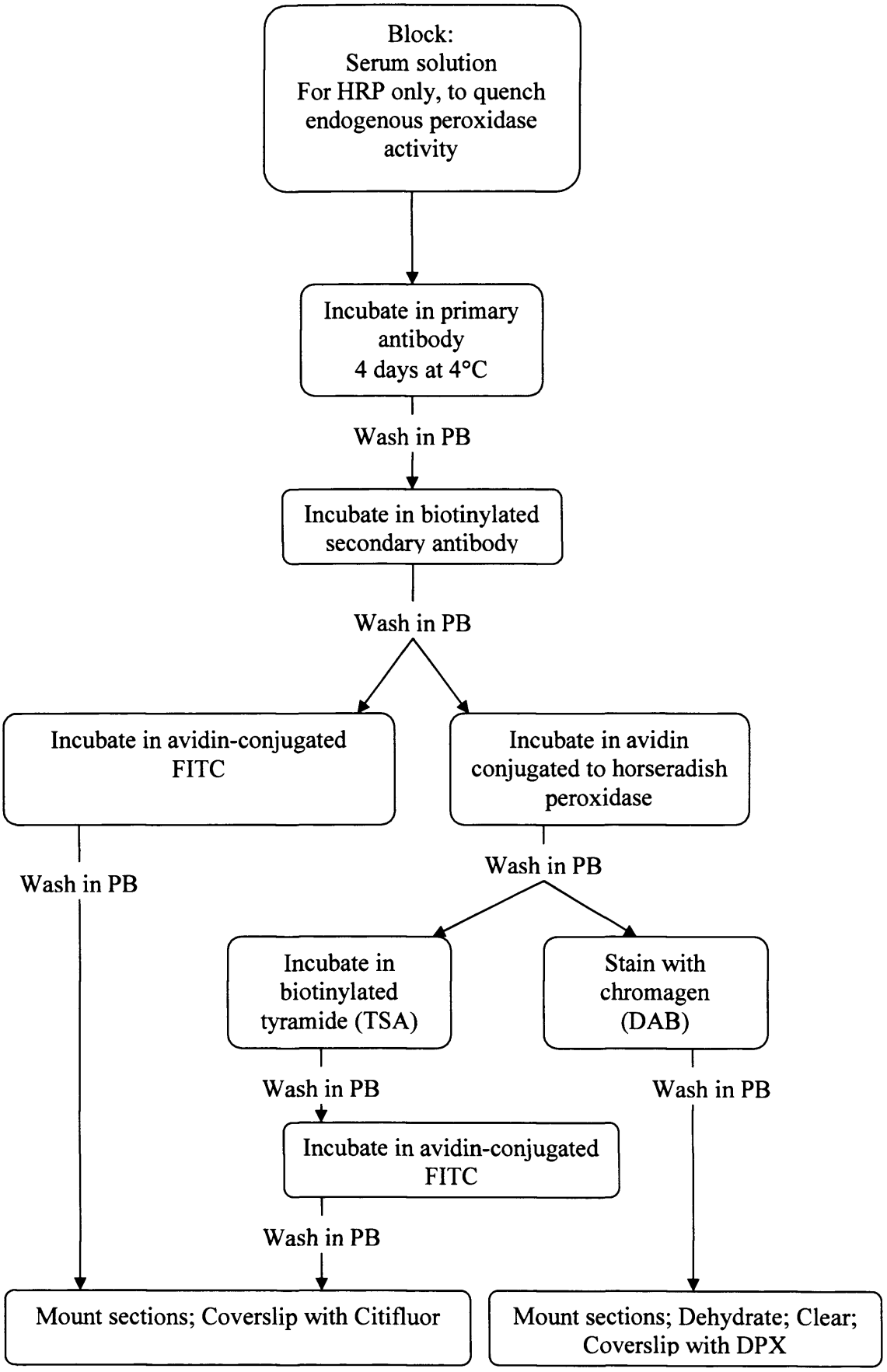


FIGURE 2.11: Step-by-step schematic of the IHC used in this thesis (Adapted from Gadd, 2003).

2.8.2.2 Primary antibody labelling

The IHC in this thesis used the avidin-biotin complex method. Avidin, a large glycoprotein found in egg-white can bind four molecules of the small molecular weight vitamin, biotin, with high affinity. The increased sensitivity results from the large number of biotin molecules that can be attached to an antibody (Guesdon *et al.*, 1979; Hsu & Raine, 1981; Hsu *et al.*, 1981). Because of the large number of biotin molecules attached to the antibody, many labelled avidin molecules may be bound at the site of the primary antigen-antibody reaction. This enables greater sensitivity to be achieved in terms of the amount of label finally bound to the antigenic site (Polak and Noorden, 1997).

Cy3 and fluorescein isothiocyanate (FITC)

Fluorescence provides a visible label which contrasts well with a dark, non-fluorescent background. FITC emits a bright apple-green fluorescence (λ 520 nm) at an excitation wavelength of 495 nm. Cy3 emits a red fluorescence and belongs to the group of CyDyes^{TM}, (Amersham International, UK).

Horseradish peroxidase (ABC) method

The instability of immunofluorescence, with time, prompted development of a more permanent type of preparation using enzymes as labels. Horseradish peroxidase is an enzyme which causes hydrogen peroxide (H_2O_2) to decompose to water and oxygen. When an electron donor is present, this peroxidase activity oxidises the donor. The chromagen and electron donor used in these studies was 3'3'-diaminobenzidine (DAB). Degradation of H_2O_2 results in oxidation of DAB. The reaction product is a brown polymer which is insoluble in water or alcohol.

Tyramide Signal Amplification (TSA)

Further amplification can be achieved using TSA. This reaction involves horseradish peroxidase catalysing the deposition of biotinylated tyramide onto the complex bound to the primary antibody. The numerous biotin molecules deposited by this reaction are then visualised indirectly using an avidin-bound label step. Use of TSA allows lower concentrations of primary antibody to be used, thereby increasing signal : background ratio on tissue sections.

The method for IHC with chromogenic detection using DAB is found in protocol 2.8.1 (see Appendix II). Details for conventional fluorescence detection are given in protocol 2.8.2 (see Appendix III), and details for TSA with fluorescent detection is found in protocol 2.8.3 (see Appendix IV).

2.8.3 Microscopy and photography

A Leica DMR (Leica Microsystems) or Nikon Eclipse E800 microscope (Nikon, Kingston upon Thames, UK) was used to visualise labelled antibody sections. Bright-field conditions were used to visualise antigens labelled chromogenically, whilst fluorescent dyes were observed under appropriate excitation from a 50 W mercury lamp (HBO50; Osram, Munich, Germany). Bright-field digital photography was carried out using a JVC colour Video camera (KY-F50; JVC, London, UK) and fluorescent photography using a Hamamatsu Chilled CCD camera (C5985; Hamamatsu Photonics, Welwyn Garden City, UK) attached to a Macintosh PowerPC G3 computer running Vision Explorer VA 1.11 (Graftek Imaging, Austin, USA).

2.8.4 General laboratory solutions

Phosphate buffer (PB; 0.1M; pH 7.4):

190 mM NaH_2PO_4 (BDH, Poole, UK)
810 mM Na_2HPO_4 (BDH, Poole, UK)

Triton Tris buffered Saline (TTBS)
0.05 M Tris saline pH 7.4
0.3 % Triton X-100
0.02 % Azide; Only if diluting antibody, as acts as a preservative of the fluorescence.

Tris buffer (0.15M; pH7.6):

127 mM Tris HCl (Sigma, Poole, UK)
23 mM Tris base (Sigma)

Heparinised phosphate-buffered saline (PBS):

Saline (0.9 % NaCl; Baxter, Lessines, Belgium)
5 ml heparin (Monoparin; CP Pharmaceuticals, Wrexham, UK)
~5 mM PB

Normal Goat Serum (NGS) solution:

0.1 M PB
3 % NGS (Vector Laboratories, Burlingame, USA)
0.3 % Triton X-100 (BDH)
0.02 % NaN_3 (Sigma)

Store at 4 °C

2.9 WESTERN BLOT ANALYSIS OF THE NORADRENALINE TRANSPORTER AND α_{2a}-ADRENOCEPTOR PROTEIN

Although IHC is a useful method for visualising receptors, transporters or enzymes, further quantification using Western blot analysis was required. Western blot analysis consists of three main steps: separation of the protein of interest by electrophoresis; protein transfer by electroblotting; and immunodetection of the individual proteins.

2.9.1 Tissue preparation

Western blot analysis of α_{2a}-adrenoceptors was performed on tissue sections from the frontal cortex, hippocampus and LC of NK1-/- and NK1+/+ mice. These cortical areas were chosen as they receive dense noradrenergic innervation from the LC. The fresh brain was quickly dissected, then frozen immediately in liquid nitrogen. Tissue was stored at -20°C, until use.

2.9.2 Method

Tissue samples were rapidly homogenised in 1 ml of RIPA buffer, containing a cocktail of proteinase and phosphatase inhibitors (1 % NP40, 20 mM Hepes pH 7.4, 100 mM NaCl, 100 mM sodium fluoride (NaF), 1 mM sodium orthovanadate (Na_3Vo_4), 10 µg / ml leupeptin and pepstatin1 µg / ml; Sigma) based on that described by Sambrook *et al.*, 1989; Harlow and Lane, 1990). RIPA buffer homogenises the protein as well as protecting, linearising and exposing the antigen. The sample is homogenised gently until a continuous suspension is generated and then the homogenate is transferred to a clean centrifuge tube. Foaming of the sample reflects significant protein denaturing. This is avoided by slow homogenisation and taking care not to lift pestle out of the solution. The samples are then boiled for 5 min so that proteins are denatured and dissolved.

Homogenates were left on ice for 2 h and then centrifuged (12,000 *x g*, 4°C) for 15 min. Centrifugation clears the lysate of insoluble proteins, carbohydrates and lipids. The clear supernatant is removed, taking care to avoid the pellet and any lipids that remain on the surface, as excess insoluble matter will block the passage of protein during SDS-PAGE (polyacrylamide gel electrophoresis). The lysate is stable for several months at -20°C.

The protein contents of the supernatant were determined using a BCA kit (Pierce, Rockford, IL) using bovine serum albumin (BSA; Pierce, Rockford, IL) as a standard.

All of the samples were mixed 50:50 with 1*X* loading buffer (10 % w/v sodium dodecyl sulphate; 50 % v/v glycerol and 0.5 % w/v bromophenol blue; Sigma) and boiled for 5-10 min to denature proteins. A total of 15 µl (10 µg total protein) of each sample was separated on a commercially available 10 % SDS polyacrylamide gel PAGEr (Bio-Rad) at 100 V for 90 min. The separated proteins were electrophoretically transferred to a hydrophobic polyvinylidene difluoride (PVDF) membrane (Bio-Rad) at 4 °C for 45 min at 100 V.

To block non-specific antibody binding, the PVDF membranes were immersed overnight at 4 °C in 4 % bovine serum albumin (BSA) and PBS / 1 % Tween. The PVDF membranes were then incubated with anti-goat α_{2a}-adrenoceptor (Santa Cruz

Biotechnology Inc.) or anti-rabbit noradrenaline transporter (NAT) antibodies (Chemicon) at a dilution of 1:1000 in 4 % BSA and PBS / 1 % Tween. All membranes were incubated with the primary antibody overnight at room temperature.

The membranes were then washed with Tris-buffered saline / 1 % Tween for 2 days, if incubated with the α_{2a}-adrenoceptor antibody, or 1 day if incubated with NAT antibody. The secondary antibody which must be against the species in which the primary antibody was raised was then applied: 1:2000 made in 4% BSA and PBS / 1% Tween. For the α_{2a}-adrenoceptor the secondary antibody was anti-goat, and for NAT the secondary antibody was anti-rabbit. After exposure to the secondary antibody the membranes are washed in PBS / Tween (6 x 5 min washes), and then left for 30 mins in PBS / Tween. This is followed by a 1 x 10 min wash in PBS. The protein bands were identified by chemiluminescence using the enhanced chemiluminescence (ECL) detection system and X-ray film for 1-20 min (Amersham Biosciences Buckinghamshire, UK).

To ensure protein was evenly loaded in the gel, the house-keeper protein, GAPDH, was used as a control. To identify GAPDH, the membranes received 2 x 10 min washes in stripping buffer (1.5 % glycine, 1 % sodium dodecyl sulphate and 1 % Tween) followed by 2 x 5 min washes in PBS followed by a block in 4 % BSA for 1 h. Membranes were subsequently incubated overnight at room temperature in GAPDH (1:750). This was followed by 6 x 5 min washes in PBS / Tween. Membranes were incubated in 1:2000 diluted horseradish peroxidase-conjugated mouse anti-rabbit IgG (Amersham Biosciences Inc., Buckinghamshire, UK).

2.10 Quantitative [^{3}H]RX821002 and [^{3}H]rauwolscine autoradiography of α_2-adrenoceptors

2.10.1 Background

Autoradiography is a process by which the location of radioactive materials in tissue sections is detected, by exposure to a photographic emulsion, forming a pattern on the film corresponding to the location of the radioactive compounds within or attached to the cell. Since the late 1800s dramatic progress in the development of this technique has been made (Table 2.2). In 1974 Kuhar and Yamamura combined radioligand binding with autoradiography to map the distribution of neurotransmitter receptors (Kuhar and Yamamura, 1974). These early studies used *in vivo* labelling. This involved labelling receptors in intact tissues by systemic administration of the radioligand. However, this technique is associated with serious limitations for example resolution, sensitivity (Stumpf, 2002), lipid solubility and penetration of the blood-brain barrier of the injected radioligands and, therefore, was followed by *in vitro* labelling. This involved incubating slide-mounted tissue sections with radioactive ligands, and enabled receptor labelling under closely controlled conditions (Young, III & Kuhar, 1979b; Young, III & Kuhar, 1979a). Adrenoceptors were first identified using light-microscope autoradiography in 1980 (Young, III & Kuhar, 1980).

History of autoradiography	
1867	Niepce de St. Victor observes blackening of silver iodide and silver chloride emulsions by uranium nitrate
1869	Bequerel observes the same darkening with opaque paper placed between the uranium nitrate and the emulsions.
1924	The first biological experiment involving autoradiography traces the distribution of polonium in biological specimens.
1943	Autoradiographs prepared by exposing lantern slides are used to trace ^{131}I in thyroid sections.
1946	The resolution of these autoradiographs is improved by painting slide emulsion directly onto the tissue sections. This technique would later form the basis for modern microradiography.
1950	The stripping of emulsion from slides leads to the development of stripping film, which resolves to light microscopic levels.
Late 1950s	^{131}I-Labeled trifluoroiodomethane is used to measure rat cerebral blood flow, despite the high volatility of the tracer compound, and the low resolution of the resulting images. When the tracer is replaced with ^{14}C-labeled antipyrine, permeability across the blood-brain barrier is reduced.
1977	^{14}C-Labeled iodoantipyrine yields accurate measurements of cerebral blood flow, and becomes the reference diffusible tracer for this experiment.
Late 1970s	Sokoloff *et al.* use [^{14}C]deoxyglucose to measure the metabolic rate of glucose.
1980s	^{14}C- and ^{3}H-labeled leucine are used to measure rates of amino acid incorporation into proteins. The use of autoradiography is also proposed for the measurement of ligand binding and drug interaction.

TABLE 2.2: History of the development of autoradiography.

Radioactive decay results in the emission of subatomic particles capable of ionizing matter. β-decay is the most common type of radiation used in autoradiography. β-decay occurs when a neutron decomposes into a proton and electron. The most commonly

used nuclides for autoradiography include ^{14}C and ^{3}H. The half-life of ^{3}H is 12.4 years better resolution is obtained with ^{3}H due to low β energies.

2.10.2 General principles of binding

The general procedures of radioligand receptor binding were developed through the use of membrane preparations. Radioligands and transmitters possess both hydrophilic and hydrophobic portions and, therefore, interact with many sites on the membranes through ionic and non-ionic bonds, producing a high degree of non-specific binding (NSB). Unless radioligands have a high enough specific activity to permit assay at concentrations as low or lower than the dissociation constant, the concentration of radioactive ligand which must be added to produce measurable radioactivity in the membranes may saturate specific receptors and label primarily non-specific sites (Snyder, 1978). Neurotransmitter receptor binding assays have had practical ramifications in the design of new drugs (Snyder, 1978).

Using membrane preparations, different types of information have been obtained from radioligand binding studies depending on the experimental conditions. Information about the affinity of a ligand for a receptor (K_d, equilibrium dissociation constant) and the density of binding sites (B_{max}) is obtained from saturation binding experiments. Tissue sections are incubated in a range of concentrations of radioligand. Total binding is assumed from the amount of radioactivity bound. NSB is determined (in parallel) by incubating tissue sections in the same conditions but including an excess (i.e. enough to saturate specific binding sites) of a suitable cold ligand. NSB is the proportion of radioligand binding which is not displaced by drugs specific for the receptor. NSB may result from binding to other receptor types, other proteins in the membrane or partitioning into lipids. Adrenaline was used as the cold ligand in the present studies: this will compete with the radioligand for binding to the α_2-adrenoceptor under investigation. Specific binding is then calculated by subtracting NSB (in the presence of adrenaline) from total binding.

Competition ligand binding assays provide information about the concentration of competing ligand which displaces 50 % of the specific binding of the radioligand (IC_{50}). In this type of assay, a single concentration of radioligand is used. The radioligand is

used at a low concentration, usually at or below its K_d value. Specific binding of the radioligand is then determined in the presence of a range of concentrations of competing cold compounds, in order to measure the potency with which they compete for the binding of the radioligand. The K_i (a measure of the affinity of the competitor for the receptor) is the concentration of competing ligand which roughly occupies 50 % of the receptors if no radioligand were present. The IC_{50} value for a compound can vary between experiments depending on radioligand concentration and amount of the target binding site etc. The K_i is an absolute value derived from the IC_{50} using the Cheng-Prusoff equation:

$$K_i = \frac{IC_{50}}{1 + ([L]/K_d)}$$

Where [L] = the concentration of free radioligand used in the assay, and K_d = the dissociation rate of the radioligand for the receptor.

Association and dissociation rate constants are determined using association and dissociation kinetic experiments for the receptor-ligand complex.

The development of autoradiography in whole brain slices, rather than membranes, has offered a useful method to study localization of the receptor of interest. Consequently anatomical information is also provided. Another advantage of autoradiography is that only small quantities of radiolabelled drug are required, which can be readily and accurately measured.

An important consideration when designing radioligand binding experiments is the choice of a radiolabelled agonist or antagonist. G protein-coupled receptors, such as α- and β-adrenoceptors, form different conformations depending on whether GTP or GDP is bound see Section 2.11. Agonists bind to the high affinity conformational states of the receptor only. However, if the concentration of the agonist is high enough, they will also bind to the low affinity conformational state of the G protein-coupled receptor. This leads to different measures of the receptor population. Antagonists will bind to both the low affinity and high affinity conformational states of the G protein-coupled

receptor. Use of a radiolabelled antagonist, therefore, gives a better estimate of the total receptor population than a radiolabelled agonist.

2.10.3 The photographic process

Autoradiographic film consists of a flexible base, onto which the photosensitive emulsion is coated, and a protective supercoat. The photographic emulsion is composed of silver halide grains dispersed within gelatine. The grains are typically ≥ 1 μm in diameter; large grains facilitate greater sensitivity, while small grains enable finer resolution. The grains consist of silver, bromide, and iodide ions arranged in a crystal lattice structure. The labelled sections are apposed to the emulsion. Charged electrons and positrons produced by β-decay attract the silver ions of the grains, this reduces the atomic silver which forms clumps, called latent image centres. When a threshold number of latent image centres is reached within a single silver halide grain, the grain is developable. Developable grains form the dark areas of the image when the emulsion is developed.

For the autoradiographic studies conducted in this thesis we used a Hyperfilm-^{3}H (Amersham Biosciences). This is a speciality, single emulsion coated, film, with no anti-scratch layer, for the direct autoradiography of ^{3}H. The anti-scratch layer on most films seriously impedes or totally blocks weak β-particles produced by tritium decay. As the hyperfilm used in these studies has no anti-scratch layer, these weak particles can strike the emulsion directly to form the image decreasing the path length of β particle emission (Figure 2.12).

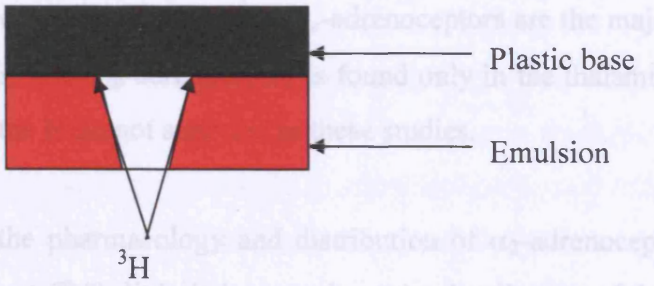


FIGURE 2.12: Hyperfilm™ ^{3}H structure.

2.10.4 Choice of radiolabel

A number of radioligands are available for labelling α_2-adrenoceptors (Table 2.3)

α_2-adrenoceptors	α_{2a}-adrenoceptors	α_{2c}-adrenoceptors
[^{3}H]RX821002[1] [^{3}H]RS79948-197[2] [^{3}H]idazoxan [3,4,5] [^{3}H]UK14304[5]	[^{3}H]RX821002[2,6]	[^{3}H]MK912[2] [^{3}H]rauwolscine[2,4]

TABLE 2.3: Commercially available radioligands available for autoradiographic labelling of α_2-adrenoceptors. Compounds underlined are agonists.

[1](Holmberg *et al.*, 2003), [2](Uhlen *et al.*, 1998), [3](Mallard *et al.*, 1992), [4](Winzer-Serhan *et al.*, 1997b), [5](Zilles *et al.*, 1993) [6](Wikberg-Matsson *et al.*, 1995)

[^{3}H]RX821002 was chosen for these studies. Previous studies have demonstrated that it labels all α_2-adrenoceptors, with some studies suggesting a preference for the α_{2a}-adrenoceptor subtype (Uhlen *et al.*, 1998). In an attempt to distinguish between the different α_2-adrenoceptor subtypes, [^{3}H]rauwolscine was also used, as this compound preferentially labels α_{2c}-adrenoceptors (Uhlen *et al.*, 1998). It was hoped that subtraction of the autoradiographic labelling with [^{3}H]rauwolscine from the autoradiographic labelling with [^{3}H]RX821002 would provide an estimate of the α_{2a}-adrenoceptor receptor population. Labelling of the α_{2b}-adrenoceptor was not thought necessary as the α_{2a} and α_{2c}-adrenoceptors are the major adrenoceptor subtypes in the CNS. The α_{2b}-adrenoceptor is found only in the thalamus (Scheinin *et al.*, 1994) an area of the brain not analysed in these studies.

Although the pharmacology and distribution of α_2-adrenoceptors is well documented within the rat CNS, little is known about the distribution of these receptors in the CNS of the mouse. [^{3}H]RX821002 autoradiography was, therefore, used to map the localisation and density of α_2-adrenoceptors in relevant brain regions in NK1+/+ and NK1-/- mice.

2.10.5 Methods

2.10.5.1 Drugs and chemicals

[^{3}H]RX821002 (specific activity 41.0 Ci / mmol) used at a concentration of 1.0 nM and [^{3}H]rauwolscine (specific activity 66.0 Ci / mmol) used at a concentration of 0.4 nM were purchased from Amersham Biosciences. (-)Adrenaline, was obtained from Sigma.

2.10.5.2 Animals and tissue preparation

Receptor autoradiography typically uses fresh-frozen specimens, as ligand binding to receptors can be destroyed by chemical fixation due to the cross linking of binding proteins. Three mice of each genotype were used. After decapitation, brains were rapidly dissected and frozen by immersion into cold isopentane chilled to 45 ± 5 °C on dry ice. Brains were than stored at -70 °C until sectioned. Sections (15 μm) were cut on a cryostat (Leica) at -22 °C in the horizontal plane. The sections were thaw-mounted onto gelatin-coated slides, dried at room temperature for 2 h, and subsequently stored at -70 °C with desiccant in sealed containers.

2.10.5.3 Autoradiographic labelling of α_2-adrenoceptors with [^{3}H]RX821002 and [^{3}H]rauwolscine

Slides with brain sections were brought to room temperature over a 15 min period. [^{3}H]RX821002 (1.0 nM) and [^{3}H]rauwolscine (0.4 nM) were made in 50-mM potassium phosphate buffer, pH 7.4. Tissue sections were incubated with 1 ml of either [^{3}H]RX821002 or [^{3}H]rauwolscine at room temperature. In each experiment sections from each genotype were processed in parallel. For [^{3}H]RX821002 (1.0 nM) the incubation time was 20 min, followed by 2 x 2 min washes in the same buffer on ice. For [^{3}H]rauwolscine (0.4 nM), the incubation time was 60 min followed by 2 x 30 min washes in ice-cold buffer. The slides were than dipped in ice-cold water to remove salts and dried under a stream of cold air.

Receptor density was assessed at the approximate K_d (single concentration point) of [^{3}H]RX821002 (1.0 nM) and [^{3}H]rauwolscine (0.4 nM) for α_2-adrenoceptor and

α_{2c}-adrenoceptors, respectively. The K_d is the concentration capable of saturating approximately half of the α_2-adrenoceptor binding sites in the brain area of interest. The use of a single concentration point simplified analysis. Also, saturation analysis cannot be reliably performed in brain areas possessing low amounts of the receptors of interest.

NSB of the radioligands was determined by parallel incubation of serial (1:5) sections in 100 μM cold (-)adrenaline, to block the binding of [^{3}H]RX821002 to α_2-adrenoceptors. Radiolabelled, dried tissue sections were apposed to tritium sensitive film (Hyperfilm [^{3}H], Amersham Biosciences) along with [^{3}H] microscales (Amersham Biosciences). After exposure for 8 weeks the films were manually developed using Kodak D-19 developer (5min) and Amfix fix (5min).

The identification and nomenclature of brain structures was based on the rat brain atlas of Paxinos and Watson (1998).

2.10.5.4 Autoradiographic image analysis

Binding was quantified using image analysis. We used a MicroComputer Imaging Device (MCID). Here a video camera captures images from an X-ray film, illuminated by a constant light source. A digital image, made up of pixels of the labelled section, is produced. Pixels possess both spatial and grey value level characteristics. For the autoradiographic studies in this thesis, results were quantified using densitometric analysis. The densitometry results are expressed as grey levels (a series of shades ranging from black to white) and converted to units of optical density. Optical density can be converted to radioactivity per mass area by construction of a calibration curve. Grey values of standards of known radioactivity that have been co-exposed with the tissue sections during generation of the autoradiogram are used for calibration.

A calibration curve was, therefore, obtained by plotting optical density of [^{3}H] calibration standards, co-exposed with tissue sections, against their radioactivity. Bilateral measurements were made from sections and averaged. NSB was subtracted from total binding. NSB was not above film background, which was, therefore, regarded as NSB. The regional amounts of bound radioactivity were converted to receptor density (Geary & Wooten, 1985). The results are expressed as fmol radioligand

bound / mg wet tissue. All data reported were from the linear optical density range required for densitometric analysis.

2.11 *Quantitative autoradiography of adrenaline-stimulated α_2-adrenoceptor-mediated [^{35}S] GTPγS binding*

[^{35}S]GTPγS autoradiography provides a novel approach to detect functional G protein activity of stimulated G protein-coupled receptors (GPCRs) in brain sections. One advantage afforded by this approach is that receptor signalling can be studied in anatomically defined brain structures. However, a complicating feature is the heterogeneously distributed signal in many brain regions under basal assay conditions. Endogenous adenosine, acting via A1 receptors, to date, is the only identified factor that may contribute to this. [^{35}S]GTPγS autoradiography predominantly detects signaling of receptors that couple to the G_i class of G proteins to which the α_2-adrenoceptors belong. The discovery of novel GPCRs in the nervous system e.g. the P2Y12 receptor has been facilitated by [^{35}S]GTPγS autoradiography. It is, therefore, becoming increasingly evident that, in cryostat brain sections, the integrity of the signaling unit, which for a large number of GPCRs resides in specialized membrane microdomains, is better preserved compared to bulk membrane preparations, suggesting that constitutive receptor activity in native brain tissue may be rigorously addressed using [^{35}S]GTPγS autoradiography.

2.11.1 Background to the technique

Activation of G protein-coupled receptors increases the rate of binding of [^{35}S]GTPγS to G proteins (Lorenzen *et al.*, 1993). When the α_2-adrenoceptor is stimulated, GDP is released from the $G_{i/o}$ protein complex, allowing GTP to bind in its place. This causes dissociation of the α subunit from the βγ subunits of the heterotrimeric G protein complex and the subsequent regulation of signal transduction systems within the cell (Figure 2.13). This biochemical step can be quantified by the use of the GTP analogue [^{35}S]GTPγS which binds irreversibly to the α-subunit (Hilf *et al.*, 1989; Lazareno *et al.*, 1993). The system is normally turned off by the intrinsic GTPase activity of the α subunit hydrolysing GTP to GDP, as [^{35}S]GTPγS is a nonhydrolysable GTP analogue this is prevented (Figure 2.14). This technique therefore allows the study of

α_2-adrenoceptor activation proximal to adenylyl cyclase. As activation of G proteins is a functional consequence of G protein-coupled receptor stimulation, this assay provides a measure of functional activity.

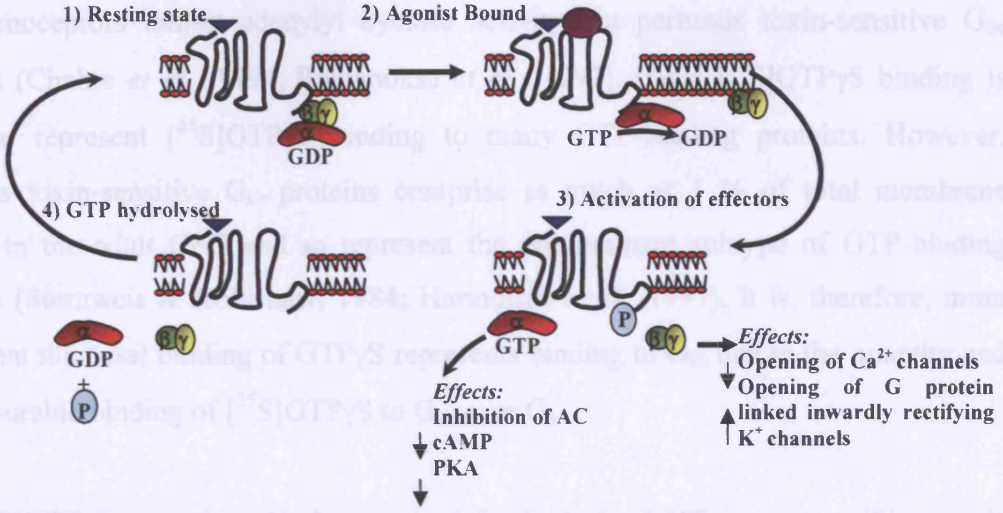


FIGURE 2.13: Coupling of the α_2-adrenoceptor to G protein. α_2-Adrenoceptors are G protein-coupled receptors. 1) G protein receptors consist of three subunits anchored to the membrane through attached lipid residues. 2) Coupling of the α-subunit to a receptor occupied by an agonist causes exchange of GDP for intracellular GTP. 3) The α-GTP complex dissociates from both the receptor and βγ complex to interact with its target, AC. The βγ complex interacts with ion channels. 4) GTP-ase activity of the α-subunit is increased once AC is bound, leading to hydrolysis of GTP to GDP, and re-association of the α-subunit with the βγ complex. AC; adenylate cyclase, cAMP; cyclic adenosine monophosphate, GDP; guanosine diphosphate, GTP; guanosine triphosphate, PKA; protein kinase A.

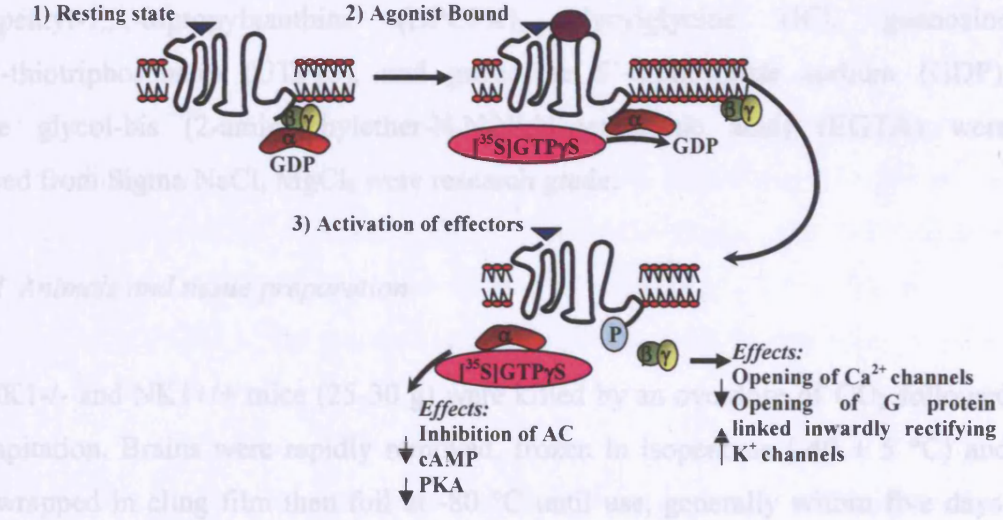


FIGURE 2.14: Schematic representation of the use of non-hydrolysable [^{35}S]GTPγS in investigating the functional activity of α_2-adrenoceptors. Agonist binding to the receptor, stimulates exchange of GDP for [^{35}S]GTPγS, which has been included in the incubating medium. In the physiological situation GTP would normally hydrolysed to GDP. As GTPγS is a non-hydrolysable form of GTP, it remains permanently bound to the activated α-subunit. This allows quantification of the functional activity of the α_2-adrenoceptor, through measurement of the radio labelled signal.

α_2-Adrenoceptors inhibit adenylyl cyclase activity via pertussis toxin-sensitive $G_{i/o}$ proteins (Chabre *et al.*, 1994; Pohjanoksa *et al.*, 1997). Basal [^{35}S]GTPγS binding is likely to represent [^{35}S]GTPγS binding to many GTP-binding proteins. However, pertussis toxin-sensitive $G_{i/o}$ proteins comprise as much as 1 % of total membrane protein in the adult CNS and so represent the predominant subtype of GTP-binding proteins (Sternweis & Robishaw, 1984; Harmouch *et al.*, 1997). It is, therefore, more likely that the basal binding of GTPγS represents binding to $G_{i/o}$ due to the quantity and the favourable binding of [^{35}S]GTPγS to $G_{i/o}$ over G_s.

The [^{35}S]GTPγS assay is well characterised for both the 5-HT_{1A} receptor (Sim *et al.*, 1995; Sim *et al.*, 1997; Waeber & Moskowitz, 1997) (Fabre *et al.*, 2000) and μ opioid receptors (Sim & Childers, 1997). The autoradiographic distribution of α_2-adrenoceptors, however, has been difficult to detect.

2.11.2 Materials

[^{35}S]GTPγS (1000-1500 Ci / mmol; guanosine 5'-(γ-thio)triphosphate was purchased from Perkin Elmer. Adrenaline bitartrate, dithiothreitol (DTT), 8-cyclopentyl-1,3,-dipropylxanthine (DPCPX), glycylglycine HCl, guanosine 5'-O-(3-thiotriphosphate) (GTPγS), and guanosine 5'-diphosphate sodium (GDP), ethylene glycol-bis (2-aminoethylether-N,N,N',N'-tetraacetic acid) (EGTA) were purchased from Sigma NaCl, $MgCl_2$ were research grade.

2.11.2.1 Animals and tissue preparation

Male NK1-/- and NK1+/+ mice (25-30 g) were killed by an overdose of CO_2 followed by decapitation. Brains were rapidly removed, frozen in isopentane (-40 ± 5 °C) and stored wrapped in cling film then foil at -80 °C until use, generally within five days. Brain sections (15 μm) were cut in the horizontal or coronal plane on a cryostat (Leica) and stored overnight with desiccant at -20 °C. For longer storage periods, sections were kept at -80 °C. Sections were brought to room temperature and air dried 30 min prior to use.

Assay buffer for adrenaline-stimulated [^{35}S]GTPγS binding assay

Assay buffer 1:

- 50mM HEPES
- 100mM NaCl
- 3mM $MgCl_2$
- 0.2mM DTT
- 0.2 mM EGTA

Assay buffer 2:

The same as assay buffer 1 but substituted with;

- 2mM GDP
- 10μM DPCPX

Assay buffer 3:

The same as assay buffer 1 and 2 but substituted with;

- 2mM GDP
- 2mM DTT
- 0.1nM GTPγS

Agonist-stimulated [^{35}S]GTPγS binding conditions were based on those previously described by (Sim *et al.*, 1995; Sim *et al.*, 1997; Waeber & Moskowitz, 1997; Fabre *et al.*, 2000) which we modified to increase signal : noise ratio (Happe *et al.*, 1999; Happe *et al.*, 2000; Happe *et al.*, 2001; Happe *et al.*, 2004; Bylund *et al.*, 2001; Bylund *et al.*, 2001; Froger *et al.*, 2001). The standard assay conditions used in this study were as follows:

Brain sections were preincubated at room temperature for an initial 15 min period in 50mM HEPES sodium salt (4-(2-hydroxyethyl)-1-piperaine ethanesulphonic acid; sigma), pH 7.5, supplemented with 100 mM NaCl, 3 mM $MgCl_2$, 0.2 mM EGTA, and 2mM DTT (assay buffer 1), and then for another 15 min in the same buffer with 2 mM GDP dilithium salt (Sigma) and 10 μM DPCPX; an A_1 adenosine receptor antagonist to decrease background labelling (assay buffer 2). Sections were then incubated for 2 h at 30 °C in the same buffer with 0.1 nM [^{35}S]GTPγS (1000 Ci / mmol) in either absence

(basal conditions) or presence (stimulated conditions) of 100 μM adrenaline-bitartrate (assay buffer 3). Adrenaline was chosen as an agonist over noradrenaline as previous studies have shown a 30-40 % lower noradrenaline-stimulated [^{35}S]GTPγS binding in the LC compared to adrenaline (Happe *et al.*, 2000). NSB was determined in the presence of 100 μM cold RX821002 to block α_2-adrenoceptors. The use of an α_2-adrenoceptor antagonist, to block adrenaline-stimulated [^{35}S]GTPγS binding, in this study demonstrates specificity of the drug action on a specific receptor type. The incubation was stopped by 2 x 2 min washes in ice-cold 50 mM HEPES, pH 7.5, and a brief immersion in ice-cold distilled water. Sections were dried under a stream of cold air and exposed to Kodak Biomax film for 24-72h. Films were developed by standard techniques 5 min in D-19 developer (Kodak, 21 °C), briefly immersed in distilled water followed by 5 min in AMFIX black and white fix. Films were analysed using a MicroComputer Imaging Device (MCID). When different film exposure times were required, film images were normalized using commercial ^{14}C standards (Amersham biosciences) calibrated for ^{35}S.

Results are expressed as mean ± s.e.m. of average measurements from n = 8 different mice. Agonist stimulated binding is expressed as percentage increase above baseline ([stimulated-basal] / basal] × 100) ± s.e.m.) based on that described by (Froger *et al.*, 2001).

2.12 Analysis of the concentration of noradrenaline in neuronal storage vesicles of NK1+/+ and NK1-/- mouse cortex

2.12.1 Tissue collection

Male (25-30 g) NK1-/- and NK1+/+ mice were killed by a blow to the head followed by cervical dislocation. Brains were removed and the cerebral cortex dissected out on ice.

2.12.2 Preparation of brain samples

2.12.2.1 Homogenisation

Cortical tissue was homogenised in 2 ml of a 0.3 M sucrose solution. Homogenate was transferred to a centrifuge tube. The homogenising tube was washed a further two times in 2 mls of sucrose solution and this solution added to the centrifuge tube.

2.12.2.2 Differential Centrifugation

The cortical homogenate was spun at 500 *x g* for 10 min, to remove undisrupted tissue. The supernatant (S_1) was decanted into clean centrifuge tubes, and temporarily stored on ice. To remove large organelles, such as nuclei and mitochondria, which form P_2, the S_1 supernatant (Figure 2.15) was then spun at 10,000 *x g* for 25 min. From this, the S_2 supernatant was decanted to a clean centrifuge tube, balanced and temporarily stored on ice. The supernatant (S_2) was then spun at 100,000 *x g* for 100 min. The final supernatant (S_3) contains NA including both soluble, cytoplasmic NA and transmitter derived from leaky or disrupted storage vesicles (Von Euler 1967). 2 ml of 0.1 M perchloric acid ($HClO_4$) was added to the P_3 pellet, which contains microsomes, including storage vesicles (Bisby & Fillenz, 1971), and the majority of NA, and homogenised briefly to precipitate the membrane proteins. 1 ml of the homogenate was then microfuged for 5 mins to compact the precipitate. The supernatant (S_4) was decanted and stored at -18 °C, before measurement of NA content by HPLC / ECD. 1 ml of 0.5 M NaOH was added to the remaining pellet and left at room temperature for estimation of protein content using the BCA method described in Section 2.9 (Figure 2.15).

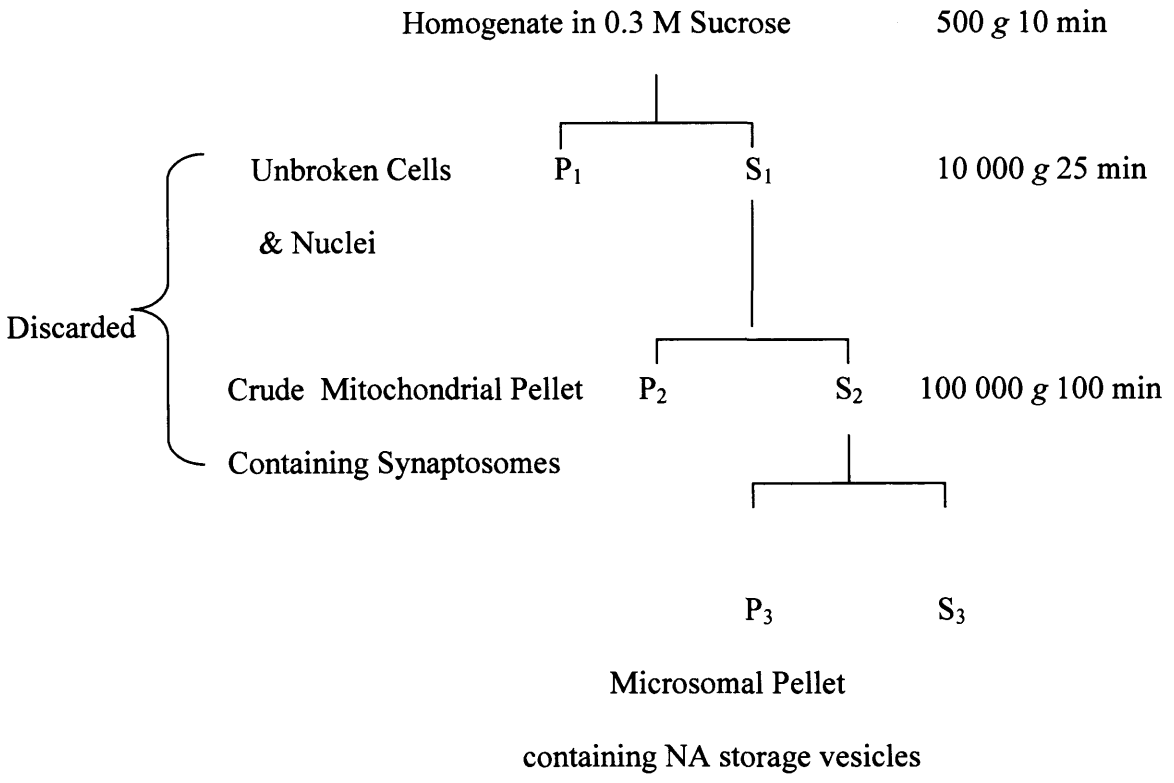


FIGURE 2.15: Pattern of differential centrifugation used for isolation of vesicular noradrenaline in these studies

2.12.3 Measurement of noradrenaline

A 1:10 dilution of the supernatant from each sample was made and 50 µl of the supernatant introduced to the HPLC microinjector. NA was analysed using HPLC / ECD as described in Section 2.4-5.

2.12.4 Estimation of protein content

This was achieved using BCA as described in Section 2.9.

2.13 STATISTICS

Details of the statistical analysis used for the studies contained in this thesis can be found in the protocol section of each chapter.

CHAPTER 3

EFFECT OF α_2-ADRENOCEPTOR ANTAGONISTS AND NORADRENALINE REUPTAKE INHIBITORS ON NORADRENALINE EFFLUX IN ANAESTHETISED NK1-/- AND NK1+/+ MICE

3 EFFECT OF α_2-ADRENOCEPTOR ANTAGONISTS AND NORADRENALINE REUPTAKE INHIBITORS ON NORADRENALINE EFFLUX IN ANAESTHETISED NK1-/- AND NK1+/+ MICE

3.1 INTRODUCTION AND OVERVIEW

Experiments presented in this chapter were aimed at replicating and extending pilot microdialysis studies conducted in this laboratory. These pilot studies, conducted under continuous halothane anaesthesia, demonstrated a 2-fold higher basal noradrenaline (NA) efflux in NK1-/- mice compared to their NK1+/+ counterparts. These results could be explained in a number of ways, as changes in the concentration of extracellular transmitter, found by microdialysis, can be attributed to either an increase in NA release, inhibition of reuptake, or both. Microdialysis measures changes in efflux which is highly regulated by both α_2-adrenoceptors and the noradrenaline transporter (NAT). One way to distinguish between these processes is through application of either a noradrenaline reuptake inhibitor, for example desipramine (DMI), or administration of an α_2-adrenoceptor antagonist, for example RX821002. Local administration of DMI would inhibit clearance of released NA from the synaptic cleft, whereas systemic administration of the α_2-adrenoceptor antagonist, RX821002, would block the autoreceptor and consequently increase firing-rate of locus coeruleus (LC)-noradrenergic neurones and release of NA. Administration of both DMI and RX821002 would provide a more realistic indication of the full potential for release and were, therefore, used in the experiments described here to investigate the underlying mechanisms of increased NA efflux in NK1-/- mice.

3.1.1 Basal noradrenaline efflux in halothane-anaesthetised NK1-/- mice

3.1.1.1 Effect of α_2-adrenoceptor antagonists

α_2-Adrenoceptors are located on both the cell bodies and terminals of noradrenergic neurones where they act to control LC noradrenergic neuronal firing and release of NA, respectively (Aghajanian *et al.*, 1977; Cedarbaum & Aghajanian, 1976) (Figure 3.1).

The hypothesis for the present studies is that the difference in basal NA efflux, between the two genotypes, is mainly due to a downregulation or desensitisation of these α_2-autoreceptors. To this end, the effect of systemic administration of the α_2-adrenoceptor antagonist, RX821002, on NA efflux was compared in the two genotypes, to provide a better insight into the function of somatodendritic and terminally located α_2-adrenoceptors (Figure 3.2; see Section 1.5 for a detailed description of why RX821002 was chosen for these studies). If the function of the α_2-adrenoceptor is impaired in the NK1-/- mice, we would predict that systemic administration of RX821002, which would block the activation of α_2-adrenoceptors located on both the cell bodies and terminals, would lead to a greater incremental increase in NA efflux in NK1+/+ mice (Figure 3.2).

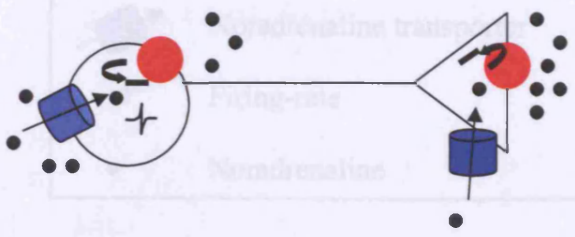


Figure 3.1: *α_2-Adrenoceptors under normal resting conditions.* The concentration of extracellular noradrenaline (NA) depends on a balance between activity-dependent exocytotic NA release, its reuptake into nerve endings via the NAT and tonic activation of autoregulatory α_2-adrenoceptors. α_2-Adrenoceptors exert control over release of NA at nerve terminals and neuronal firing-rate at the level of cell bodies of the LC.

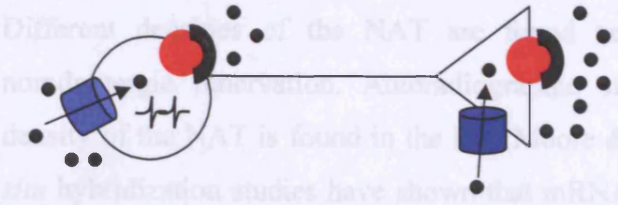
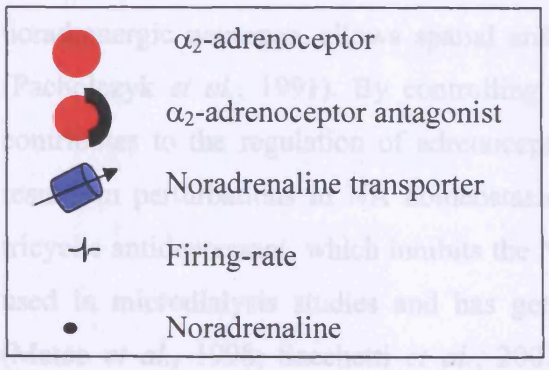


FIGURE 3.2: *Systemic administration of RX821002.* Systemic RX821002 antagonises α_2-adrenoceptors located on the cell body and terminals and so increases cell firing and release of NA. However, reuptake of NA by the NAT may mask any differences in release.



Herpfer *et al.*, (2005), by infusing RX821002 (1.0 μM and 10 μM) into the frontal cortex, investigated terminal control of NA release (Figure 3.3). Following RX821002 infusion, NA efflux was increased in both genotypes, suggesting that tonic activation of α_2-adrenoceptors, located in the terminal field, constrains the spontaneous release of NA in both NK1+/+ and NK1-/- mice.

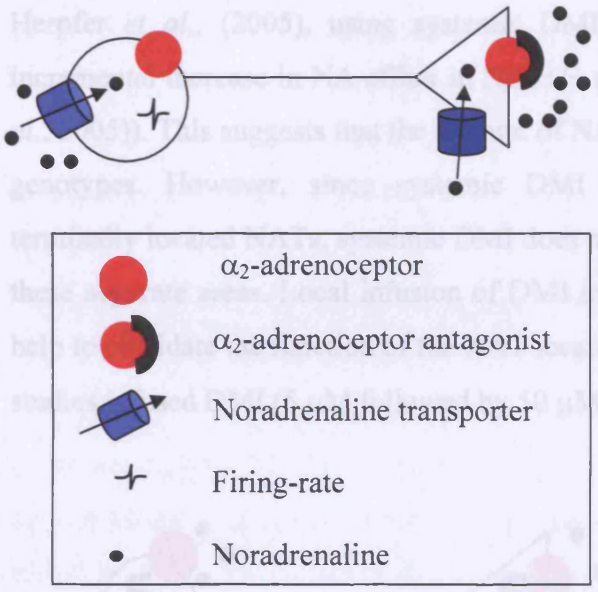


FIGURE 3.3: In the studies by Herpfer *et al.*, (2005) infusion of RX821002 into the frontal cortex antagonises α_2-adrenoceptors located on the terminals, increasing release of NA. Any change in extracellular efflux of NA would provide evidence for the tonic inhibitory influence of α_2-adrenoceptors in the terminal field of the two genotypes. However, reuptake of NA by the NAT may diminish any differences in release.

3.1.1.2 Effect of noradrenaline reuptake inhibition on noradrenaline efflux in NK1+/+ and NK1-/- mice

Different densities of the NAT are found across different brain areas receiving noradrenergic innervation. Autoradiographic studies have shown that the highest density of the NAT is found in the LC (Moore & Bloom, 1979; Tejani-Butt, 1992). *In situ* hybridization studies have shown that mRNA for the NAT is found exclusively in noradrenergic neurones (Lorang *et al.*, 1994). Selective expression of the NAT on noradrenergic neurones, allows spatial and temporal control over the actions of NA (Pacholczyk *et al.*, 1991). By controlling the availability of synaptic NA, the NAT contributes to the regulation of adrenoceptor signalling; disruption of the NAT gene results in perturbations in NA homeostasis (Xu *et al.*, 2000). DMI is a well known tricyclic antidepressant, which inhibits the Na^+ / Cl^- dependent NAT. DMI is commonly used in microdialysis studies and has generally been shown to increase NA efflux (Mateo *et al.*, 1998; Sacchetti *et al.*, 2001; Beyer *et al.*, 2002; Garcia *et al.*, 2004;

Fernandez-Pastor *et al.*, 2005). Since the DMI-induced increase in NA efflux is constrained by both somatodendritic and terminally located autoregulatory α_2-adrenoceptors, acting to control release of NA or firing-rate of LC neurones (Mateo *et al.*, 1998; Dennis *et al.*, 1987), the increase in NA efflux will depend on the tonic rate of NA release.

Herpfer *et al.*, (2005), using systemic DMI (10 mg / kg i.p.), showed the same incremental increase in NA efflux in NK1+/+ and NK1-/- mice (Figure 3.4; (Herpfer *et al.*, 2005)). This suggests that the amount of NA cleared by the NAT is the same in both genotypes. However, since systemic DMI inhibits both somatodendritically and terminally located NATs, systemic DMI does not distinguish the function of the NAT at these separate areas. Local infusion of DMI into either the frontal cortex or LC would help to elucidate the function of the NAT located at these areas. Therefore, these current studies infused DMI (5 μM followed by 50 μM) into the terminal field (Figure 3.5).

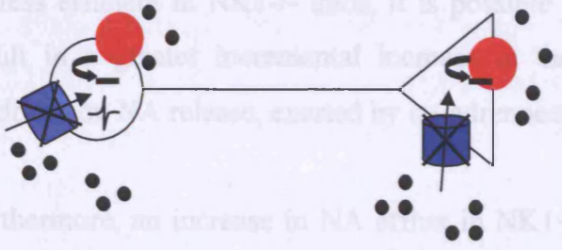


FIGURE 3.4: ***Systemic administration of DMI inhibits the NAT on the cell body and terminals.*** **Consequently both α_2-adrenoceptors located on the cell bodies and terminals are activated preventing a further rise in extracellular NA. Systemic DMI increases NA efflux to the same extent in both NK1+/+ and NK1-/- mice (Herpfer *et al.*, 2005)**

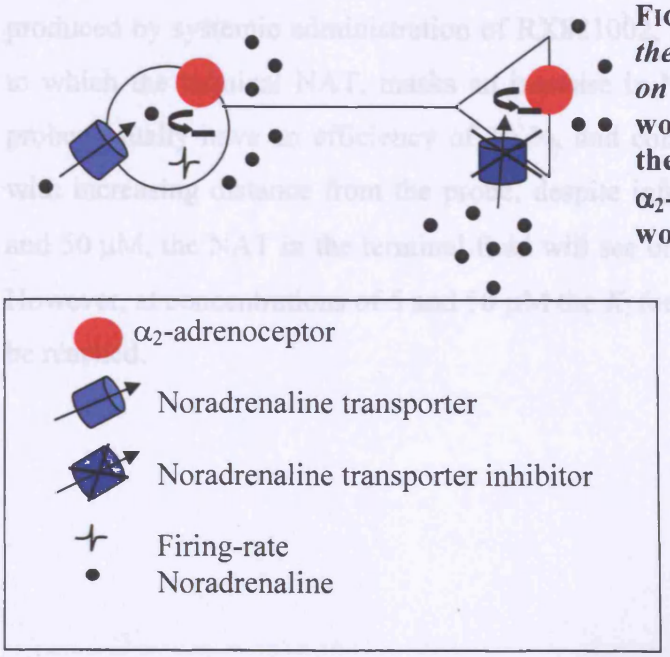


FIGURE 3.5: ***Infusion of DMI into the frontal cortex inhibits the NAT on noradrenergic terminals.*** **This would increase extracellular NA in the terminal field. However, α_2-adrenoceptors on the terminals would constrain this release.**

The rise in terminal NA following local DMI infusion will activate terminal α_2-adrenoceptors constraining further release of NA. However, somatodendritic α_2-adrenoceptors are unlikely to be affected leaving firing-rate unchanged (Figure 3.5). Systemic administration of DMI, alone, is likely to produce an increase in NA at the level of both the cell bodies and terminal regions, consequently activation of α_2-adrenoceptors, located somatodendritically and at terminal regions, will suppress both firing-rate of LC neurones and release of NA, preventing a further increase in NA efflux and possibly even leading to a decrease in NA efflux (Figure 3.4). It is, therefore, possible that local cortical infusion of DMI may result in a larger increase in NA efflux, compared to systemic administration of DMI.

Since local DMI would block the uptake of released NA in the terminal field, only, we would expect to see an increase in NA efflux in both NK1+/+ and NK1-/- mice. This increase in NA efflux may be constrained by the terminal autoregulatory α_2-adrenoceptors in NK1+/+ mice (Figure 3.5). As we believe the α_2-adrenoceptors to be less efficient in NK1-/- mice, it is possible that DMI infusion into the cortex may result in a greater incremental increase in these animals due to impaired negative feedback on NA release, exerted by α_2-adrenoceptors.

Furthermore, an increase in NA efflux in NK1+/+ or NK1-/- mice following systemic administration of an α_2-adrenoceptor antagonist may be masked by sequestration of NA through the NAT. Comparison of the results from local infusion of DMI, with those produced by systemic administration of RX821002, alone, should determine the extent to which the terminal NAT, masks an increase in NA release. Because microdialysis probes usually have an efficiency of 10 %, and concentrations of perfusates decrease with increasing distance from the probe, despite infusing DMI at a concentration of 5 and 50 μM, the NAT in the terminal field will see only a fraction of this concentration. However, at concentrations of 5 and 50 μM the K_i for the rat NAT (7.36 nM) is likely to be reached.

3.1.1.3 Combining systemic α_2-adrenoceptor antagonists and local noradrenaline reuptake inhibition in NK1+/+ and NK1-/- mice

Combining α_2-adrenoceptor antagonists with noradrenaline reuptake inhibitors, and altering their routes of administration (systemic versus cortical infusion), enables a clearer insight into differences in the rate of release between NK1+/+ and NK1-/- mice. Blockade of the NAT means that possible differences in the rate of release of NA are no longer masked by clearance through the NAT. Invernizzi & Garattini, (2004) and Nutt *et al.*, (1997) combined systemic administration of a noradrenaline reuptake inhibitor, e.g. DMI, with systemic administration of an α_2-adrenoceptor antagonist e.g. RX821002 and demonstrated a marked enhancement of the increase in extracellular NA induced by the noradrenaline reuptake inhibitor. However, these experiments give no indication of whether the potentiation of NA efflux is through blockade of somatodendritic α_2-adrenoceptors, located on noradrenergic neurones in the LC and / or terminal α_2-adrenoceptors. In the studies performed by Herpfer *et al.*, (2005) infusion of RX821002 (0.1 and 1.0 μM) via retro-dialysis, into the frontal cortex, increased NA efflux to a greater extent in DMI-pre-treated NK1+/+ mice, than in NK1−/− mice. DMI pre-treatment did not augment the increase in NA induced by RX821002 infusion in NK1−/− mice. These findings suggest that terminal α_2-adrenoceptors are functioning differently in NK1+/+ and NK1-/- mice.

If α_2-adrenoceptors are downregulated or desensitised in NK1-/- mice, then combining systemic administration of an α_2-adrenoceptor antagonist should have little, or no effect, on the incremental increase in NA efflux following cortical infusion of DMI. In contrast, in NK1+/+ mice α_2-adrenoceptors should constrain any increase in NA efflux following DMI infusion. As a consequence combining an α_2-adrenoceptor antagonist systemically with local DMI should result in a greater incremental increase in NA efflux in NK1+/+ mice, compared with NK1-/- mice (Figure 3.6) as seen previously (Herpfer *et al.*, 2005).

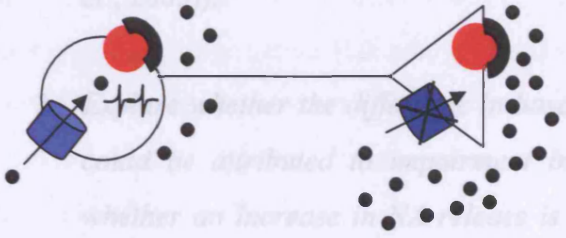


FIGURE 3.6: ***Systemic administration of RX821002 in combination with DMI infused into the frontal cortex.*** **RX821002 antagonises α_2-adrenoceptors located on the cell body and terminals, increasing cell firing and release of NA. A difference in the rate of release should no longer be masked by reuptake in the frontal cortex and cause a marked enhancement of NA efflux.**

3.1.2 Aims

The experiments described in this chapter were designed to complement those performed by (Herpfer *et al.*, 2005). The main aim was to provide evidence that the α_2-adrenoceptor mediated regulation of NA release is impaired in NK1-/- mice.
The objectives were to:

1. *Confirm the preliminary findings, in anaesthetised mice, that NK1-/- mice display higher basal efflux of NA in the frontal cortex when compared with their wildtype counterparts* (Fisher *et al.*, 2003.,2004; Stewart *et al.*, 2004;(Herpfer *et al.*, 2005)).

2. *Explore whether the difference in basal NA efflux, in NK1+/+ and NK1-/- mice could be attributed to impairment in the function of α_2-adrenoceptors, and whether an increase in NA release is masked by clearance through terminally located noradrenaline transporters.*

This involved determining the effect of systemic administration of RX821002, alone, on NA efflux in NK1+/+ and NK1-/-mice. Systemic administration of RX821002 would inhibit both somatodendritic α_2-adrenoceptors which govern cell firing, and terminal α_2-adrenoceptors, which govern release. However, reuptake of NA by the NAT in the terminal field could mask increased release of NA, following antagonism of the α_2-adrenoceptors, alone. To determine the contribution made by the terminal NAT to clearance of NA, NA efflux, following local infusion of DMI into the frontal cortex, was monitored in both NK1+/+ and NK1-/- mice. By combining systemic administration of RX821002, with local infusion of DMI into the terminal field, thereby excluding the influence of clearance through the NAT, we were further able to determine the full potential for NA release governed by α_2-adrenoceptors.

3.2 PROTOCOLS

All procedures complied with the Animals (Scientific Procedures) Act 1986. Adult (6-8 weeks, 25-30g) male NK1-/- and NK1+/+ mice (n=4) for ***experiment 1*** and (n=9) for ***experiment 2*** were used. Probes were implanted as described in Section 2.6.1. Experiments started at 08:00 a.m. every day.

3.2.1 Experiment 1: Systemic administration of RX821002 alone

NK1+/+ and NK1-/- mice were divided randomly into groups destined for either saline or RX821002 treatment. Once a stable baseline had been achieved, RX821002 (0.3 mg / kg i.p.) or saline was administered, and sampling continued for a further 240 min. [This dose was chosen based on preliminary pilot studies. At the 10-fold higher dose of 3 mg / kg i.p. the mortality rate in NK1-/-, but not NK1+/+ mice, was unacceptable].

3.2.2 Experiment 2: Systemic RX821002 in combination with intracortical DMI infusion

NK1+/+ and NK1-/- mice were divided randomly into two groups and given one of the following treatments:

- Systemic administration of saline followed by cortical DMI infusion at 5 μM and 50 μM.
- Systemic administration of RX821002 (0.3 mg / kg i.p.) followed by cortical infusion of DMI at 5 μM and 50 μM.

Three basal samples were collected to ensure a stable baseline, RX821002 (0.3 mg / kg i.p.) was administered and a further 2 x 20-min samples were collected. Ringer's solutions containing 5 μM and 50 μM of DMI were then infused, via retro-dialysis, 40 min after injection of RX821002, at which time the NA efflux response to RX821002 had stabilised (see ***experiment 1***). 3 x 20-min dialysis samples were collected, at each concentration (Figure 3.7).

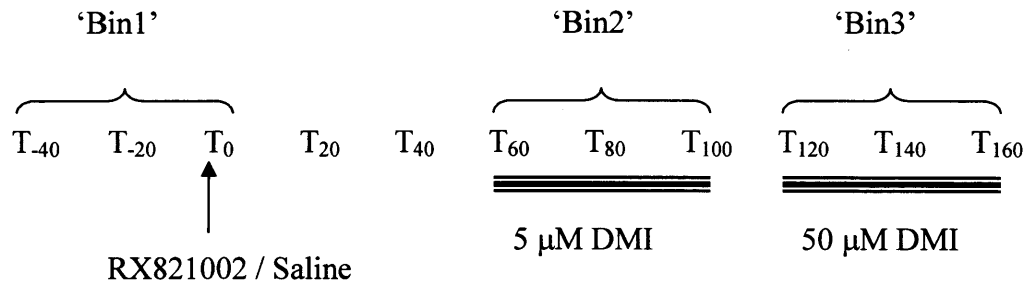


FIGURE 3.7: Schematic of the dosing protocol and the definition of the 'bins' used in analysis of the data.

The concentration of NA in the dialysate samples was determined using HPLC / ECD as described in Sections 2.4-2.5. Data are expressed as mean ± s.e.m. fmol / 20 min noradrenaline concentration, without correction for probe recovery.

3.2.3 Data Analysis

Statistical analysis was performed on both raw and net changes in efflux. Net values were calculated by subtracting the mean of three consecutive 'basal' samples from all the dialysis samples of an individual animal see: (Dalley *et al.*, 1996). When comparing drug-induced changes and basals, statistical analysis of the raw data was carried out. All microdialysis data were analysed using 3-way repeated measures ANOVA in SPSS PC$^+$. 'Time' was considered a 'within subjects' factor. When looking for genotype dependent effects of drug, 'drug treatment' and 'genotype' were the between subjects factors. If an overall effect of 'time', 'drug treatment', 'genotype', or an interaction between them, was found, using multifactorial ANOVA, data for a single factor were further investigated using least significant difference (LSD) multiple comparisons post-hoc analysis. This allowed us to identify differences between the different groups. When efflux was compared with basal levels, the analysis was carried out in bins of three consecutive samples.

For these studies:

- 'bin1' = Basal samples (T_{-40}-T_0)
- 'bin2' = 5 μM DMI (T_{60}-T_{100})
- 'bin3' = 50 μM DMI (T_{120}-T_{160})

See Figure 3.7 for the schematic of the 'bins' used in these studies.

If data violated the Mauchley's test for sphericity, then the Greenhouse-Geisser 'ε' correction was used. A value of $\mathbf{P \leq 0.05}$ was required for statistical significance.

3.3 RESULTS

3.3.1 Experiment one: Effect of systemic RX821002 on noradrenaline efflux

Between T_0-T_{120} following either RX821002 (0.3 mg / kg i.p.) or saline (10 ml / kg i.p.) there was a main effect of 'genotype' ($F_{1,12}$ = 16.52; P = 0.002; T_{20}-$T_{120;}$ Figure 3.8a-b) and a 'genotype x drug' interaction ($F_{1,12}$ = 6.04; P = 0.03; T_{20}-T_{120}), demonstrating a drug dependent change in NA efflux, which was different between genotypes. For 180-min after systemic administration of either RX821002 (0.3 mg / kg i.p.) or saline (10 ml / kg i.p.) a main effect of 'genotype' ($F_{1,12}$ = 13.57; P = 0.003; T_{20}-T_{180}) was still observed but the 'drug x genotype' interaction just failed to match the criterion for significance ($F_{1,12}$ = 4.67; P = 0.052).

3.3.1.1 Basal efflux differed between NK1-/- and NK1+/+ mice

Mean basal efflux in the frontal cortex of NK1-/- mice was 8.99 ± 2.2 fmol / 20 min. This was higher than basal efflux (1.73 ± 0.23 fmol / 20 min) in the NK1+/+ mice ($F_{1,15}$ = 19.69; P = 0.001) (Figure 3.8a-b).

3.3.1.2 Effect of saline injection on NK1+/+ and NK1-/- mice

Systemic administration of 0.9 % saline (10ml / kg i.p.), at $T_{0,}$ had no effect on NA efflux in either NK1-/- ($F_{2,6}$ = 1.56; P = 0.29) or NK1+/+ ($F_{1,3}$ = 2.99; P = 0.19) mice, over the 240 min of the experiment (Figure 3.8a). Although, in NK1-/- mice, there was an apparent increase in NA efflux after the injection of saline, this did not differ from baseline. The higher NA efflux in NK1-/- mice, compared to NK1+/+ mice, was maintained throughout the experiment ($F_{1,6}$ = 19.84; P = 0.007; T_0-$T_{240;}$ 'genotype'; Figure 3.8a).

3.3.1.3 RX821002 (0.3 mg / kg i.p.) increased noradrenaline efflux in NK1+/+ mice only

Post-hoc analysis was performed over the T_0-T_{120} min post-injection of RX821002. This revealed that RX821002 increased NA efflux in the frontal cortex of NK1+/+ only, compared to basals, and abolished the difference in NA efflux in NK1+/+ and NK1-/- mice (Figure 3.8b). After treatment with RX821002, the NA efflux in NK1+/+ mice, was not different from that in NK1-/- mice treated with RX821002 (P = 0.278; Figure 3.8b). NA efflux in NK1+/+ mice after RX821002 (0.3 mg /kg i.p.), was greater than NK1+/+ mice treated with saline (P = 0.024; Figure 3.8d) but not different from NK1-/- mice treated with saline (P = 0.064). RX821002 had no effect on NA efflux in NK1-/- mice (Figure 3.8c).

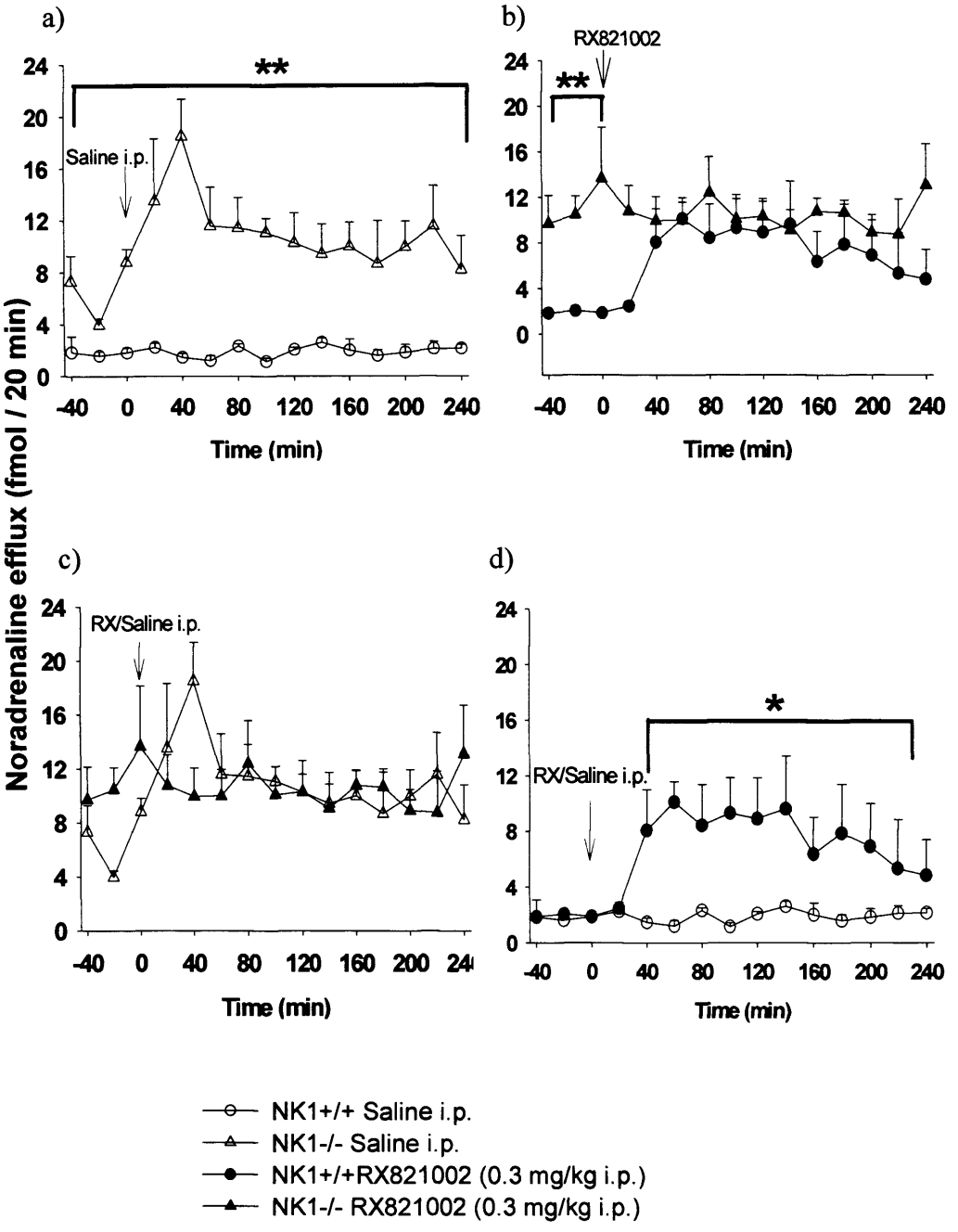


FIGURE 3.8a-d: Effect of RX821002 (0.3 mg / kg i.p.) on noradrenaline efflux in halothane-anaesthetised NK1+/+ and NK1-/- mice. RX821002 increased noradrenaline efflux in NK1+/+ mice, only. This increase abolished the genotype dependent difference in cortical noradrenaline efflux. Values are expressed as mean ± s.e.m. n = 4. * = P ≤ 0.05; ** = P ≤ 0.001.

3.3.2 Experiment two: Effect of intracortical infusion of desipramine on RX821002 induced increase in noradrenaline efflux

This experiment examined NA efflux in four groups:

- **Groups 1 and 2:** Systemic administration of saline followed by cortical DMI infusion at 5 μM and 50 μM *in both NK1+/+ and NK1-/- mice.*
- **Groups 3 and 4:** Systemic administration of RX821002 (0.3 mg / kg i.p.) followed by cortical infusion of DMI at 5 μM and 50 μM *in both NK1+/+ and NK1-/- mice.*

The results describe, first, whether RX821002 increases NA efflux over the 40 min pre-treatment time. Groups 1 and 2 are then analysed to determine the effect of DMI infusion, alone, on NA efflux in NK1+/+ and NK1-/- mice. The results then describe the comparison between all four groups to determine if pre-treatment with RX821002 potentiates the DMI induced increase in NA efflux. The following results are from one fully randomised experiment.

3.3.2.1 Effect of genotype on basal noradrenaline efflux

As with experiment 1 (Figure 3.8), there was a marked difference in basal NA efflux in NK1+/+ and NK1-/- mice. Mean basal efflux in the frontal cortex of NK1-/- was 6 ± 1.5 fmol / 20 min and this was higher than the mean basal efflux of 2 ± 1 fmol / 20 min in the NK1+/+ mice ($F_{1,28} = 6.06$; $P = 0.02$ 'genotype'; Figure 3.9a-b).

Over the 40 min post injection of RX821002, analysis of the raw data showed that NA efflux differed with genotype ('time x genotype' interaction; $F_{3,76} = 3.03$ $P = 0.036$; T_0-T_{40}; Figure 3.9b-c), and that the increase in NA efflux was dependent on the drug ('time x drug' interaction; $F_{3,76} = 4.18$; $P = 0.009$) in both genotypes. Analysis of the net change in NA efflux from T_0-T_{40} post-injection of RX821002 at T_0, revealed a 'genotype x drug' interaction ($F_{1,27} = 7.3$; $P = 0.012$ T_0-T_{40}; Figure 3.10b-c), demonstrating an effect of RX821002 on NA efflux which differed between genotypes.

3.3.2.2 RX821002 pre-treatment increases cortical noradrenaline in NK1+/+ mice, only

LSD post-hoc analysis showed that RX821002 increased the net efflux of NA in NK1+/+ mice, only (Figure 3.10b-c). The net increase in NA efflux in NK1+/+ mice was greater than that in NK1-/- mice ($P = 0.001$) following treatment with RX821002, and, greater than that in saline treated NK1+/+ ($P = 0.006$) and NK1-/- ($P = 0.005$) mice. No difference between individual groups was revealed by LSD post-hoc analysis of raw data (Figure 3.9b-c).

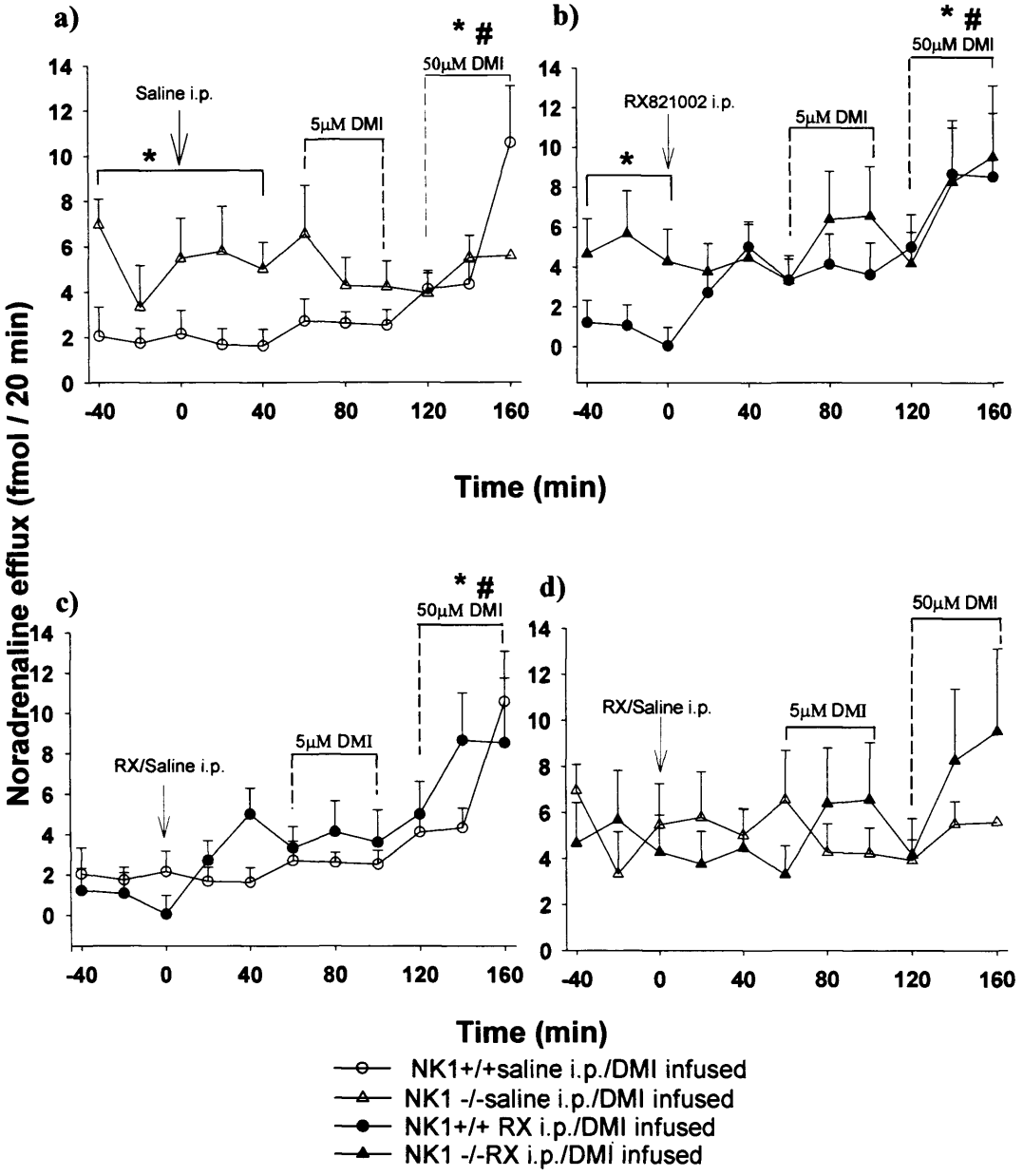


FIGURE 3.9a-d: Raw data. Effect of DMI infused into the frontal cortex alone, and combined with RX821002 pre-treatment in NK1+/+ and NK1-/- mice. RX821002 (0.3 mg / kg i.p.) increased noradrenaline concentration in NK1+/+ mice, only. DMI increased noradrenaline efflux at a concentration of 50μM only, and only in NK1+/+ mice. Pre-treatment with RX821002 did not potentiate the increase in noradrenaline efflux in either genotype.Values are mean ± s.e.m. $ = $P \leq 0.05$ c.f NK1-/-; ** = $P \leq 0.05$ c.f. basal; # = $P \leq 0.05$ c.f. 5 μM; n = 9.

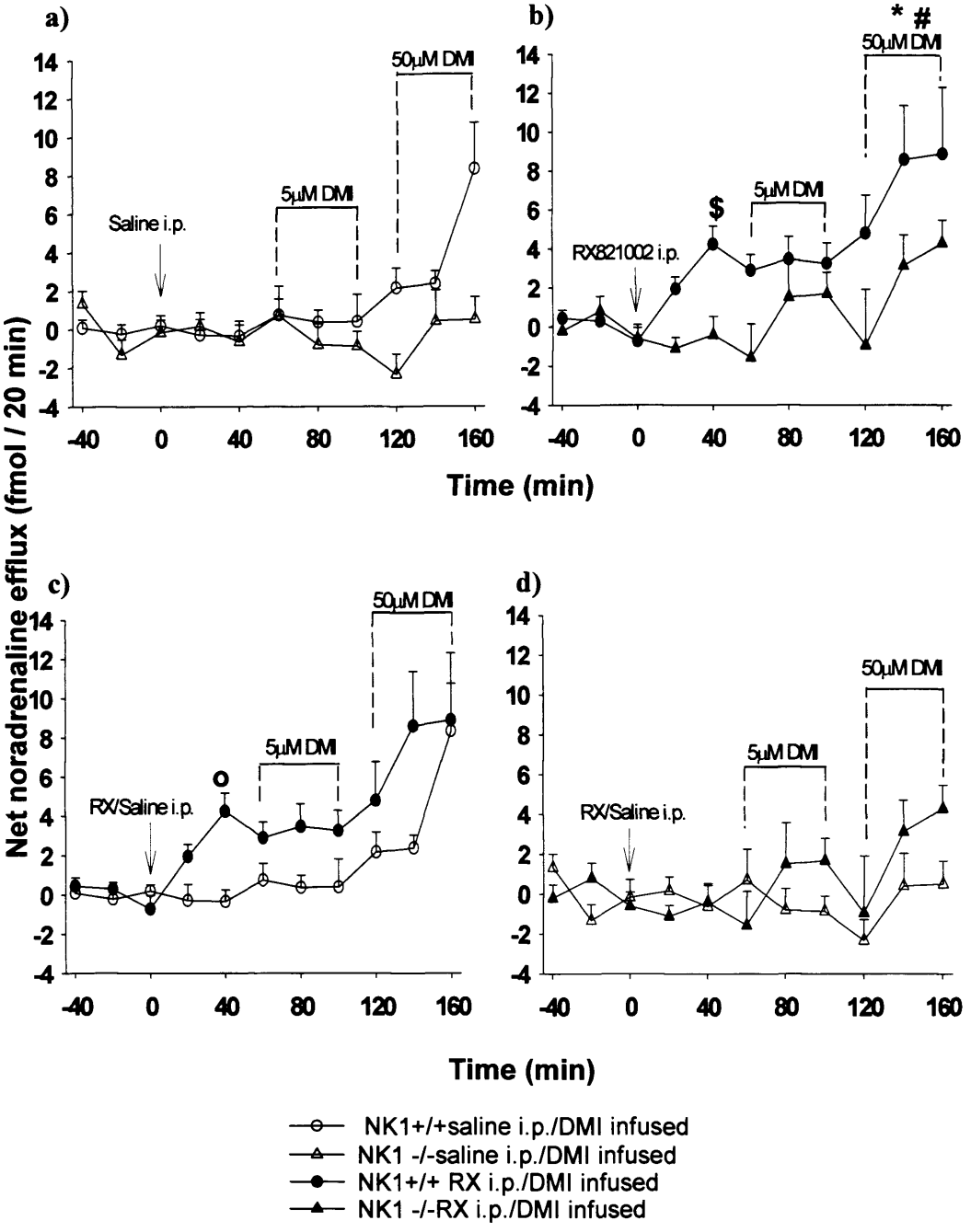


FIGURE 3.10a-d: Net noradrenaline efflux. Effect of DMI infused into the frontal cortex alone and combined with RX821002 pre-treatment in NK1+/+ and NK1-/- mice. RX821002 (0.3 mg / kg i.p.) increased noradrenaline concentration in NK1+/+ mice, only. DMI increased noradrenaline efflux at a concentration of 50μM only, and only in NK1+/+ mice. Pre-treatment with RX821002 did not potentiate the increase in noradrenaline efflux in either genotype.Values are mean ± s.e.m. * = $P \leq 0.05$ c.f. basal; # = $P \leq 0.05$ c.f. 5 μM; $ = $P \leq 0.05$ c.f. NK1-/-; o = $P \leq 0.05$ c.f. saline treatment ; n = 9.

3.3.2.3 Intracortical infusion of DMI increases noradrenaline efflux in NK1+/+ mice, only

To determine the effect of intracortical infusion of DMI alone, on NA efflux, only saline pre-treated NK1+/+ and NK1-/- mice were included for this analysis.

In saline pre-treated mice, DMI increased NA efflux ($F_{3,29} = 3.04$; $P = 0.041$; T_{-40}-T_{160}; 'time'), but this differed across genotypes ($F_{3.27,29.46} = 2.91$; $P = 0.047$; T_{-40}-T_{160}; 'time x genotype' interaction). Infusion of 5 μM DMI had no effect in either genotype (Figure 3.9 and 3.10). However, in NK1+/+ mice only, NA efflux was increased by 50 μM DMI compared to both basals and 5 μM DMI (Figure 3.9 and Figure 3.10). See footnote[1] for statistics used to support this conclusion.

LSD post-hoc analysis did not reveal a difference between the groups. However, analysis of saline pre-treated NK1+/+ mice, only, showed that 50 μM DMI 'bin3', increased NA efflux compared to basals 'bin1' ($F_{1,7} = 20.97$; $P = 0.003$) and 5 μM DMI 'bin2' ($F_{1,6} = 18.05$; $P = 0.005$). There was no effect of DMI at any concentration in saline pre-treated NK1-/- mice.

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[1] *Raw data:*

'Bin1' (basals; T_{-40}-T_0) versus 'bin3' (50 μM DMI; T_{120}-T_{160}): $F_{1,11} = 5.91$; $P = 0.033$.

'Bin x genotype' interaction: $F_{1,11} = 7.09$; $P = 0.022$.

LSD post-hoc analysis did not reveal a difference between groups.

'Bin2' (5 μM DMI, T60-T100) versus 'bin3' (50 μM DMI, T_{120}-T_{160}): $F_{1,10} = 5.64$; $P = 0.039$.

'Bin x genotype' interaction: $F_{1,10} = 10.47$; $P = 0.009$.

3.3.2.4 *Systemic administration of RX821002 followed by cortical infusion of DMI does not potentiate noradrenaline efflux in either genotype*

To determine if pre-treatment with RX821002, potentiated the DMI induced increase in NA efflux compared with saline pre-treatment, all treatment groups were included in this analysis.

Analysis of the *raw data* showed that NA efflux increased with time across all groups: main effect of 'time' ($F_{3,55} = 5.59$; $P = 0.002$; T_{60}-T_{160}; Figure 3.9a-d). Analysis of the *net increase* in NA efflux, showed that this increase in NA efflux was different between genotypes ($F_{4,71} = 2.6$; $P = 0.043$; T_{60}-T_{160}; 'time x genotype'; Figure 3.10a-d).

Analysis of the *raw data* showed that infusion of 50 μM DMI increased NA efflux compared to 5 μM DMI: 'bin2' (5μM DMI, T_{60}-T_{100}) versus 'bin3' (50μM DMI, T_{120}-T_{160}): $F_{1,19} = 7.24$; $P = 0.014$; main effect of 'bin'. This main effect of 'bin' was different between NK1+/+ and NK1-/- mice ('bin x genotype' interaction: $F_{1,19} = 6.11$; $P = 0.023$). However, no difference between groups was found using LSD post-hoc analysis.

Analysis of the *net data* also showed a main effect of 'bin' ($F_{1,19} = 7.24$; $P = 0.014$) and an overall 'bin x genotype' interaction ($F_{1,19} = 6.11$; $P = 0.023$). LSD post-hoc analysis showed no increase in NA efflux in NK1-/- mice pre-treated with either saline or RX821002 over the entire experiment. LSD post-hoc analysis revealed the net increase in NA efflux caused by 50 μM DMI in RX821002 pre-treated NK1+/+ mice was greater than both saline ($P = 0.001$) and RX821002 ($P = 0.049$) pre-treated NK1-/- mice. Net NA efflux induced by 50 μM DMI was not different between RX821002 and saline pre-treated NK1+/+ mice, showing that pre-treatment with RX821002 did not potentiate the increase in NA efflux when DMI was infused into the frontal cortex, suggesting release rate was maximal already.

3.4 DISCUSSION

3.4.1 Main findings from these studies

3.4.1.1 Genetic disruption of the NK1 receptor increases noradrenaline efflux in halothane-anaesthetised NK1-/- mice

Basal NA efflux in anaesthetised NK1-/- mice was 4-5 fold higher than the NK1+/+ counterparts, supporting previous studies in this laboratory (Fisher *et al.*, 2003; Stewart *et al.*, 2004; Herpfer *et al.*, 2005).

3.4.1.2 Systemic RX821002 increases noradrenaline efflux in NK1+/+ mice, only

Systemic administration of the α_2-adrenoceptor antagonist, RX821002 (0.3 mg / kg i.p.), increased NA efflux in the frontal cortex of halothane-anaesthetised NK1+/+ mice, only, and the genotype-dependent difference in NA efflux was abolished. Systemic administration of RX821002 would antagonise α_2-adrenoceptors at the level of cell bodies in the LC and on terminals. A reduction in α_2-adrenoceptor inhibitory function would be expected to increase NA release and blunt the response to drugs acting at these receptors in NK1-/- mice. Therefore, the lack of effect of RX821002 in NK1-/- mice can be attributed to an impaired function of either somatodendritically or terminally located α_2-adrenoceptors. The RX821002-induced increase in NA efflux in NK1+/+ mice is in agreement with studies in rats (Meana *et al.*, 1997; Hudson *et al.*, 1999). The increase in NA efflux in NK1+/+ mice, only, following systemic RX821002, suggests that reuptake through the terminal NAT, does not conceal a genotype dependent difference in the release rate of NA.

It has been suggested that NK1 antagonists attenuate the responsiveness of α_2-adrenoceptors, to systemic injection of the α_2-adrenoceptor agonist clonidine (Haddjeri & Blier, 2000). This attenuated response to clonidine after administration of an NK1 receptor antagonist may generalise to an attenuated response of α_2-adrenoceptors to the endogenous agonist NA in NK1-/- mice, and hence an increase in basal NA efflux. Attenuation of the negative feedback role exerted by

α_2-adrenoceptors in NK1-/- mice would tend to increase NA, as well as decreasing the response to systemic administration of α_2-adrenoceptor antagonists such as RX821002.

3.4.1.3 Cortical infusion of desipramine increases noradrenaline efflux in NK1+/+ mice, only

The results presented here show that a 5 μM DMI infusion does not increase NA efflux in either NK1+/+ or NK1-/- mice. Infusion of 50 μM DMI increased NA efflux compared to basal, but only in NK1+/+ mice, confirming regulation of NA release differs between the two genotypes. This suggests that although reuptake through the NAT does not mask a difference in NA release, between NK1+/+ and NK1-/- mice following systemic RX821002, some of the released NA is normally cleared by reuptake through the NAT in NK1+/+ mice, only.

The lack of effect of local cortical infusion of DMI on NA efflux in NK1-/- mice is intriguing. If the autoregulatory α_2-adrenoceptors are desensitised in NK1-/- mice, a substantial increase in NA efflux would be expected following infusion of DMI. As Stewart *et al.*, (2004) have demonstrated an increase in NA efflux when 5 μM DMI was infused into the frontal cortex, the lack of effect of DMI on NA efflux in NK1-/- mice cannot be explained by a fault with the terminal NAT.

The extracellular concentration of DMI when it is infused through the microdialysis probe is not possible to determine. This is because the drug concentration will form a gradient which decreases with increasing distance from the probe. Assuming a probe efficiency of 10 %, the concentration of DMI surrounding the probe will be well within the range of the K_i (7.36 nM; Table 3.1) for the NAT. Therefore, the lack of effect of DMI, on NA efflux in NK1-/- mice, is unlikely to be due to infusion of a concentration unable to block the NAT fully.

	pK_i Value ± s.e.m. (K_i values in parentheses)		
	Rat NAT	Human NAT	Bovine NAT
Compound			
(-)-Adrenaline	5.187 ± 0.040 (6.50 μM)	5.435 ± 0.034 (3.68 μM)	5.301 ± 0.033 (5.00 μM)
Desipramine	8.13 ± 0.091 (7.36 nM)	8.069 ± 0.046 (8.54 nM)	8.052 ± 0.023 (8.88 nM)

TABLE 3.1: pK_i and K_i values for adrenaline and DMI inhibition of noradrenaline uptake in COS-7 cells expressing the rat, human, or bovine NAT. IC_{50} values were calculated from percent inhibition of specific uptake of [3H]noradrenaline (10nM). Each IC_{50} value was converted into a K_i value according to Cheng and Prussoff method assuming competitive inhibition. Adapted from (Paczkowski *et al.*, 1999).

Using the isolated perfused rat heart two distinct mechanisms of uptake of NA have been identified (Iversen, 1965), each having characteristics of a saturable active transport system, and possess strikingly different drug sensitivities. The two distinct mechanisms are identified as *uptake 1* and *uptake 2* corresponding to neuronal and extraneuronal uptake, respectively. The DMI-sensitive NAT mediates the high affinity *uptake 1* of NA, with a low maximum rate of uptake. *Uptake 2* has a low affinity and transports adrenaline, isoprenaline, and NA at a much higher maximum rate.

It is possible that NA clearance, through *uptake 1* in NK1-/- mice, is constrained by a 'ceiling' effect, due to the low maximum rate of uptake of this transporter and the higher basal efflux of NA in NK1-/- mice. For this reason, inhibition of the NAT in NK1-/- mice may not result in an increase in NA, since the NAT is not removing a substantial amount of NA from the synaptic cleft. Competition between DMI and the high NA efflux in NK1-/- mice, for the binding site on the NAT, may also result in an increase in the IC_{50} of DMI required to inhibit the NAT in these animals.

Another reasonable explanation, for the lack of effect of DMI in NK1-/- mice, is that the higher basal NA efflux in anaesthetised NK1-/- mice means that these animals are primed for removal of NA from the extracellular space, in order to maintain a homeostatic concentration of NA. It is possible that, in NK1-/- mice, reuptake through

alternative transporters for example the dopamine (DA) or even (but less likely) the serotonin (5-HT) transporters, which belong to the same subfamily of Na^+/Cl^--dependent transporters as the NAT, masks the increase in NA efflux following local infusion of DMI. Although the DA (Giros *et al.*, 1994) and 5-HT (Pacholczyk *et al.*, 1991) transporters are not very efficient at removing NA (K_i 10 000 nM), a role for these low affinity transporters could be important in situations where there are high amounts of NA. It is possible that a developmental consequence of disruption of the NK1 receptor, and the need to remove higher amounts of NA, has meant that they have become more efficient at removing NA or are even upregulated in NK1-/- mice.

Alternatively, the extracellular NA in NK1-/- mice may be taken up through a DMI insensitive, apparently fluoxetine sensitive transporter, located at non-noradrenergic sites. Available evidence suggests that fluoxetine inhibits NA reuptake through one or more non-noradrenergic sites, possibly a transporter on dopaminergic neurones (Hughes & Stanford, 1998; Hughes & Stanford, 1996). Such a transporter could explain why DMI had no effect on NA efflux in NK1-/- mice in these current studies. It is possible that this non-noradrenergic transporter has been upregulated in NK1-/- mice, to compensate for the higher basal NA efflux observed during halothane anaesthesia. Following infusion of DMI, increased efflux in NA could be taken up through this transporter in NK1-/- mice, preventing an observable increase in NA efflux.

The cloning of the human extraneuronal transporter by Schomig and colleagues (Schomig *et al.*, 1998), marked the discovery of the second transporter responsible for *uptake 2*, which is inhibited by steroids. In humans, *uptake 2* is mediated via the extraneuronal transporter for monoamine transmitters (Grundemann *et al.*, 1998), and in the rat through the organic cation transporter (OCT3) (Wu *et al.*, 1998). This transporter is effective at removing adrenaline and NA, when catecholamine concentration increases above a critical threshold (Iversen, 1967), and binds the rodent stress hormone corticosterone with high affinity (Iversen & Salt, 1970; Grundemann *et al.*, 1998). In spite of the fact that these transporters have been well characterized *in vitro*, their *in vivo* roles remain elusive. mRNA for OCT3 has been found in neural cell populations of the rat cerebellum, hippocampus and cerebral cortex (Wu *et al.*, 1998). This could indicate that in these brain regions, OCT3 acts to inactivate the fraction of released NA that escapes *uptake 1* through the DMI sensitive NAT, and may explain why these

studies failed to see an increase in NA efflux, following infusion of DMI in NK1-/- mice.

Alternatively, the higher basal NA efflux observed in NK1-/- mice could result in an upregulation of the NAT. To maintain homeostatic NA signalling, NAT levels would be expected to decrease when synaptic NA levels are low and increase when synaptic NA levels are high, and loss of the NAT or NA clearance has been associated with depression (Klimek *et al.*, 1997). Depletion of NA, with reserpine, decreases NAT levels (Lee *et al.*, 1983), whereas increasing the concentration of NA with monoamine oxidase inhibitors, increases the number of NATs (Lee *et al.*, 1983). Although NA is not required for a downregulation of the NAT (Weinshenker *et al.*, 2002), regulation of the NAT may be more sensitive to changes in NA in younger mice (Weinshenker *et al.*, 2002). This suggests that the impact of genetic disruption of the NK1 receptor, on the noradrenergic system at early developmental stages, could result in profound changes in the regulation of the NAT in these animals.

DMI may also act on receptors non-specifically in NK1-/- and not NK1+/+ mice. DMI is an α_1-adrenoceptor antagonist. α_1-Adrenoceptor antagonists e.g. prazosin suppress the excitatory influence of the α_1-adrenoceptor on serotonergic DR neurones, decreasing 5-HT efflux in the DR (Bortolozzi & Artigas, 2003). This would lead to a decrease in the inhibitory input of the DR to the LC, which would be predicted to lead to an increase in NA efflux in NK1+/+ and NK1-/- mice. However, α_1-adrenoceptor antagonists also depress noradrenergic function (Marek & Aghajanian, 1999) and decrease both 5-HT and NA efflux in the hippocampus (Weikop *et al.*, 2004), so it may be that in NK1-/- mice α_1-adrenoceptors are more sensitive to the antagonising effects of DMI, thereby preventing an increase in NA efflux (also: Herpfer *et al.*, 2005). DMI also antagonises histamine receptors. Administration of the H_1 antagonist clorpheniramine decreases extracellular NA in the paraventricular nucleus / anterior hypothalamic region of the rat (Bealer, 1993), so we cannot exclude the possibility that in NK1-/- mice, but not NK1+/+ mice the antagonistic actions of DMI at H_1 receptors is acting to constrain the release of NA (see: (Herpfer *et al.*, 2005). DMI also antagonises muscarinic receptors. Muscarinic receptors generally inhibit NA release, and so this action is unlikely to contribute to the lack of effect of DMI in NK1-/- mice reported here.

3.4.1.4 Systemic RX821002 does not potentiate noradrenaline efflux following cortical desipramine infusion

As seen with the previous experiment, systemic RX821002 increased NA efflux in NK1+/+ mice, only. When DMI was infused through the probe a further increase was observed with 50 μM DMI, only. This increase evoked by DMI was not augmented by pre-treatment with RX821002 in either genotype.

The lack of potentiation on NA release suggests that in NK1+/+ mice, release rate is maximal following systemic pre-treatment with RX821002. This increase in release could overwhelm the NAT, so that reuptake through the NAT does not mask a difference in the release of NA, between NK1+/+ and NK1-/- mice. Therefore, although cortical infusion of DMI, alone, results in an increase in NA efflux in NK1+/+ mice, only, demonstrating that some released NA is normally cleared by the NAT. This clearance does not mask a difference in the rate of release, between NK1+/+ and NK1-/- mice, attributable to a difference in the α_2-adrenoceptor.

Although no potentiation on NA efflux in either genotype was observed by combining systemic α_2-adrenoceptor antagonism with cortical infusion of a noradrenaline reuptake inhibitor, both DMI and RX821002 were effective at increasing NA efflux in NK1+/+ mice, only. The lack of effect of RX821002 in NK1-/- mice supports the notion that α_2-adrenoceptors in these animals are not functioning efficiently. The lack of effect of DMI in NK1-/- mice may be attributed to re-uptake through alternative transporters, as described in the previous section. The lack of effect of combining systemic RX821002 and local infusion of DMI, in NK1-/- mice, may result from a combination of these factors.

3.4.2 Comparison of current findings and previous studies

Since these studies were run in parallel with those performed by Herpfer *et al.*, (2005), Table 3.2 presents a summary of the results found with these current studies in comparison with those found by Herpfer *et al.*, (2005).

Herpfer *et al.*, (2005)	NK1 +/+	NK1 -/-	Current studies	NK1 +/+	NK1 -/-
Effect on noradrenaline efflux					
Systemic DMI (10 mg / kg i.p.) alone	↑	↑	***Systemic RX821002*** (0.3 mg / kg i.p.) alone	↑	—
Infusion of ***RX821002*** (1.0 and 10.0 μM) into the frontal cortex alone.	↑	↑	***Infusion*** of ***DMI*** (50 μM) into the frontal cortex alone.	↑	—
Infusion of ***RX821002*** (0.1 and 1.0 μM) into the frontal cortex combined with ***systemic*** administration of ***DMI*** (10 mg / kg i.p.).	↑↑	↑	***Infusion*** of ***DMI*** (5 μM and 50 μM) into the frontal cortex combined with ***systemic*** administration of ***RX821002*** (0.3 mg / kg i.p.).	↑	—

TABLE 3.2: Summary of the results found by Herpfer *et al.*, (2005) and results found in these current studies.

Although in these current studies cortical infusion of DMI, did not increase NA efflux in NK1-/- mice, systemic administration of DMI (10 mg / kg i.p.), does produce the same incremental increase in NA efflux, in both NK1-/- and NK1+/+ mice (Herpfer *et al.*, 2005).

In contrast to the results presented herein, demonstrating that systemic administration of RX821002 increases NA efflux in NK1+/+ mice, only, Herpfer *et al.*, (2005) showed that local infusion of RX821002 into the terminal field, increases NA release to the same extent in both NK1+/+ and NK1-/- mice (Herpfer *et al.*, 2005). However, cortical infusion of RX821002, increases NA efflux to a greater extent in NK1+/+ mice only,

following pre-treatment with systemic DMI (Herpfer *et al.*, 2005), whereas systemic RX821002 followed by local infusion of DMI has no potentiating effect on NA efflux. The lack of a potentiating effect on NA efflux, produced by pre-treatment with RX821002, is also in contrast to a number of reports demonstrating that antagonism of α_2-adrenoceptors augments the increase in NA caused by a noradrenaline reuptake inhibitor or dual 5-HT / NA reuptake inhibitors (Dennis *et al.*, 1987; Wortley *et al.*, 1999a; Wortley *et al.*, 1999b; Geranton *et al.*, 2003).

3.4.3 Interpretation of these current findings in light of previous findings

By combining systemic administration of DMI with cortical infusion of RX821002, the studies performed by Herpfer *et al.*, (2005), focused on the terminal control of NA release. By combining systemic RX821002 with cortical infusion of DMI, the studies contained herein provide further information on the activity of both somatodendritic and terminal α_2-adrenoceptors.

Noradrenaline reuptake inhibitors, including DMI, inhibit the firing-rate of LC noradrenergic neurones by increasing the endogenous noradrenergic tone on somatodendritic α_2-adrenoceptors (Mateo *et al.*, 1998; Meana *et al.*, 1992; Palij & Stamford, 1994; Svensson & Usdin, 1978; Szabo & Blier, 2001). Therefore, it is likely that in the studies of Herpfer *et al.*, (2005), systemic DMI would limit firing-dependent NA release in the frontal cortex of NK1+/+ mice, due to increased activity at somatodendritic α_2-adrenoceptors, which decrease firing-rate (Figure 3.11).

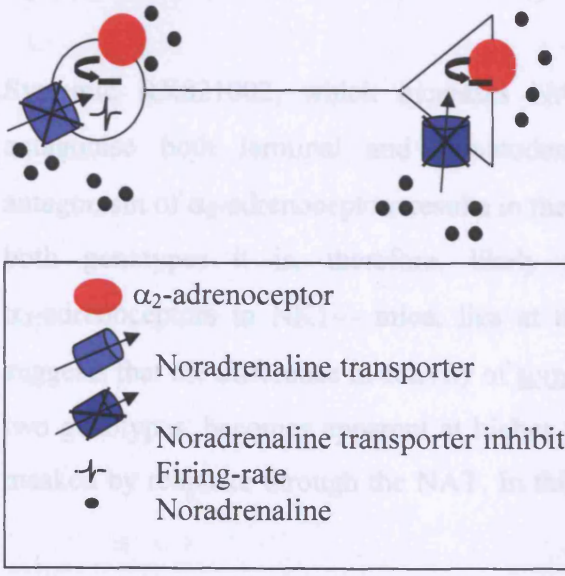


FIGURE 3.11: Systemic administration of DMI inhibits the NAT on the cell body and terminals. As a consequence both α_2-adrenoceptors located on cell bodies and terminals are activated preventing a further rise in extracellular NA.

Since it is hypothesised that the efficiency of somatodendritic α_2-adrenoceptors is decreased in NK1-/- mice, DMI is unlikely to alter the firing-rate of LC-noradrenergic neurones in these animals. The potentiated effect on NA efflux, by combining systemic DMI followed by intracortical RX821002, observed in NK1+/+ mice only, suggests that the influence of the NK1 receptor on NA release, controlled by terminal α_2-adrenoceptors, is revealed only at low rates of NA release and low firing-rates (Herpfer *et al.*, 2005). This is further supported by the lack of augmentation on NA efflux, observed when systemic RX821002, which would increase firing-rate and release of NA, is combined with local infusion of DMI in these current studies.

Furthermore, it is likely that local cortical infusion of DMI will have no effect on firing-rate of LC-noradrenergic neurones, and, therefore, will not blunt the increase in NA efflux in either NK1+/+ or NK1-/- mice. It is possible, therefore, that the lack of effect of cortical DMI in NK1-/- mice, could be due to these animals attempting to maintain homeostatic signalling, which is at a higher level, by removing excess NA through alternative transporters for example the low affinity DA, 5-HT or OCT transporters described previously.

The same incremental increase in NA efflux in both NK1+/+ and NK1-/- mice following cortical infusion of RX821002 (Herpfer *et al.*, 2005) demonstrates that terminal α_2-adrenoceptors tonically constrain efflux of NA in both genotypes. The results also suggest that clearance through the terminal NAT masks a difference in release of NA, since systemic DMI, followed by cortical infusion of RX821002, reveals a potentiated response in NA release in only NK1+/+ mice.

Systemic RX821002, which increases NA efflux in NK1+/+ mice only, would antagonise both terminal and somatodendritic α_2-adrenoceptors. Since terminal antagonism of α_2-adrenoceptors results in the same incremental increase in NA efflux in both genotypes it is, therefore, likely that the decreased tonic constraint of α_2-adrenoceptors in NK1-/- mice, lies at the level of LC cell bodies. Moreover, it suggests that the difference in activity of somatodendritic α_2-adrenoceptors, between the two genotypes, becomes apparent at higher firing-rates and rates of release, and is not masked by reuptake through the NAT. In this situation there is no augmentation of NA

efflux in NK1+/+ mice, following cortical infusion of DMI, suggesting a 'ceiling' effect on NA release.

Taken together these findings strongly indicate that the regulatory influence of both the α_2-adrenoceptor and NK1 receptor on noradrenergic neurones is revealed during different rates of LC-noradrenergic cell firing and release of NA. Potentiation of NA efflux, produced by systemic DMI and local α_2-adrenoceptor antagonism, is observed only during low firing-rates and low rates of NA release, and, only in NK1+/+ mice. This would suggest that the different activity of terminal α_2-adrenoceptors, between the two genotypes, becomes apparent at lower firing-rates. Conversely, at higher firing-rates, as would occur following systemic RX821002, NA efflux is increased in NK1+/+ mice only, and the genotype dependent difference in NA efflux is abolished. If RX821002 has no effect on somatodendritic α_2-adrenoceptors in NK1-/- mice, because they are desensitised, firing-rate in these animals will be unaffected. However, systemic RX821002 is likely to increase the firing-rate in NK1+/+ mice and this situation reveals the difference in activity of somatodendritic α_2-adrenoceptors between the genotypes. This may explain why local cortical infusion of RX821002, which would have little or no effect on firing-rate, results in the same incremental increase in NA efflux in both genotypes (Herpfer *et al.*, 2005).

This interpretation is further supported by a study revealing that stress-induced activation of *c-fos,* in the rat LC, is enhanced by NK1 receptor antagonism (Hahn & Bannon, 1998). Systemic administration of NK1 receptor antagonists alone, does not result in substantial LC *c-fos* expression (Hahn & Bannon, 1998). This suggests that the excitatory effect of NK1 receptor antagonism on LC neurones becomes apparent during situations which are likely to increase the firing-rate of LC-noradrenergic neurones. Furthermore, it suggests that the effect of genetic disruption of the NK1 receptor on the noradrenergic system, particularly the α_2-adrenoceptor, may also become more apparent during situations where the firing-rate of the LC has been artificially manipulated by drugs, as seen here, or during stressful stimuli.

3.4.4 Halothane anaesthesia as a confounding factor

Noradrenergic projections of the central nervous system (CNS) are known to be important targets in general anaesthesia (Angel, 1993). It has also been suggested that noradrenergic neurones in the LC are important targets in mediating the hypnotic actions of α_2-adrenoceptor agonists such as clonidine (De Sarro *et al.*, 1987; Scheinin & Schwinn, 1992). Anaesthetics such as sodium pentobarbitone decrease NA efflux in the frontal cortex of rats by 92 % (Dalley *et al.*, 1998). Halothane is a volatile anaesthetic. Volatile anaesthetics can also reduce NA release in the CNS and alter neuronal excitability in a non-specific way (Mizuno *et al.*, 1994).

During halothane anaesthesia, LC neurones fire in a spontaneous, slow and regular pattern. The reduction in LC firing induced by halothane may be due to a halothane induced membrane hyperpolarisation, leading to a decrease in cell firing (Nicoll & Madison, 1982). Withdrawal from halothane anaesthesia, results in an increase in LC neuronal firing (Saunier *et al.*, 1993) NA efflux (Chave *et al.*, 1996) and a 5-fold increase in the ED_{50} of intravenous clonidine (Saunier *et al.*, 1993). The increase in the effectiveness of clonidine as a sedative during halothane anaesthesia, suggests an increase in the sensitivity of α_2-adrenoceptors. Furthermore, acutely administered clonidine reduces the halothane anaesthetic requirement in dogs by a maximum of 48 % (Bloor & Flacke, 1982). This is consistent with a synergism between halothane and α_2-adrenoceptors on LC neuronal firing (Saunier *et al.*, 1993), or an increased sensitivity of α_2-adrenoceptors during halothane anaesthesia (Saunier *et al.*, 1993). The variable effects of anaesthetics makes it difficult to draw direct links to their mode of action in NK1+/+ and NK1-/- mice. Nevertheless, halothane anaesthesia exposes differences in basal NA efflux, between NK1+/+ and NK1-/- mice, which is not apparent during freely-moving microdialysis (Chapter 4).

Studies carried out in our laboratory also demonstrate that when freely-moving mice are placed under halothane anaesthesia, NA efflux is decreased in both genotypes. However, the net decrease in NA efflux is greater in NK1+/+ mice (Yan, unpublished observations). This suggests that halothane anaesthesia decreases NA efflux to a greater extent in NK1+/+ mice compared with NK1-/- mice. This may be attributed to a decrease in the activity of α_2-adrenoceptors in NK1-/- mice, thereby attenuating the

decrease in NA efflux, and further supports a difference in the activity of these receptors between these two genotypes.

3.4.5 Summary

In halothane-anaesthetised NK1-/- mice, systemic RX821002 and local infusion of DMI have no effect on NA efflux. However, in NK1+/+ mice, both RX821002 and DMI increase NA efflux. The lack of effect of RX821002 in NK1-/- mice could be the result of either a downregulation and / or desensitisation of the autoregulatory α_2-adrenoceptor. This downregulation and / or desensitisation of the autoregulatory adrenoceptor could be a cause or consequence of the 4-5 fold increase in basal NA efflux in NK1-/- mice.

3.4.6 Conclusions

- There is a difference in the regulation of NA efflux between NK1+/+ and NK1-/- mice

- This difference in the release regulating mechanism could involve a downregulation / desensitisation of autoregulatory α_2-adrencoptors. However, alterations in alternative transporters, for example the OCT, can not, as yet, be ruled out.

The following chapters compare the effect of RX821002 on freely-moving NK1+/+ and NK1-/- mice and investigate whether there is further evidence that α_2-adrenoceptors are downregulated or desensitised.

CHAPTER 4

EFFECT OF ANTAGONSIM OF α_2-ADRENOCEPTORS ON NORADRENALINE EFFLUX IN THE FRONTAL CORTEX OF FREELY-MOVING NK1+/+ AND NK1-/- MICE

4 EFFECT OF ANTAGONISM OF α_2-ADRENOCEPTORS ON NORADRENALINE EFFLUX IN THE FRONTAL CORTEX OF FREELY-MOVING NK1+/+ AND NK1-/- MICE

4.1 INTRODUCTION

In Chapter 3, it was demonstrated that the selective α_2-adrenoceptor antagonist, RX821002 (0.3 mg / kg i.p.), increased noradrenaline (NA) efflux in the frontal cortex of halothane-anaesthetised NK1+/+ mice, only, and abolished the 4-5-fold difference in basal NA efflux.

The current study was aimed at investigating the effect of antagonism of the α_2-adrenoceptor, using RX821002, on NA efflux in freely-moving NK1+/+ and NK1-/- mice. A difference in noradrenergic efflux between NK1+/+ and NK1-/- mice, in response to RX821002, would further support the hypothesis that α_2-adrenoceptors in NK1-/- mice are impaired in some way. Freely-moving *in vivo* microdialysis is preferred, as animals are free of halothane anaesthesia. Further, probes have been implanted 24 h prior to the experiment and mice have been allowed to remain in the home cage in which the experiment is conducted.

4.1.1 Background

Extensive investigations into the modulation of NA release in the cerebral cortex, with particular focus on the role of terminal α_2-adrenoceptors, have been carried out in the freely-moving rat, in this laboratory as well as others. Mateo *et al.,* found that, in the rat, NA release in cortical terminal fields is under tonic modulation by somatodendritic α_{2a}-adrenoceptors located on LC neurones (Mateo *et al.*, 1998; Mateo & Meana, 1999). This was achieved using RX821002 and the selective α_{2a}-adrenoceptor antagonist BRL44408 which were infused, via retrodialysis, into the locus coeruleus (LC). The study performed by Mateo *et al.,* (1998), found that basal NA efflux in the LC and the frontal cortex were elevated (30 %) by the local infusion of RX821002 in the LC. Additionally, at a dose of 2 mg / kg i.p. RX821002 produced a modest increase in the firing-rate of LC neurones (Mateo *et al.*, 1998) and completely reversed the inhibitory

effect of a low dose of DMI (1 mg / kg i.p.) on the firing-rate of noradrenergic neurones. RX821002 has also been shown to produce an increase in NA efflux in the bed nucleus stria terminalis (BNST) when perfused into this brain area (Forray *et al.*, 1997; Forray *et al.*, 1999), in the cingulate cortex when perfused into the LC (Mateo *et al.*, 1998; Fernandez-Pastor & Meana, 2002) and in the frontal cortex following systemic administration (Wortley *et al.*, 1999a). All this evidence suggests that there is tonic inhibitory modulation of cortical NA release by α_2-adrenoceptors located either somatodendritically, or, on neuronal terminals (Cedarbaum & Aghajanian, 1976; Aghajanian *et al.*, 1977) (Figure 4.1). Furthermore, these studies demonstrate the ability of RX821002, administered directly via retrodialysis, or systemically, to increase NA efflux in a number of brain regions in rats.

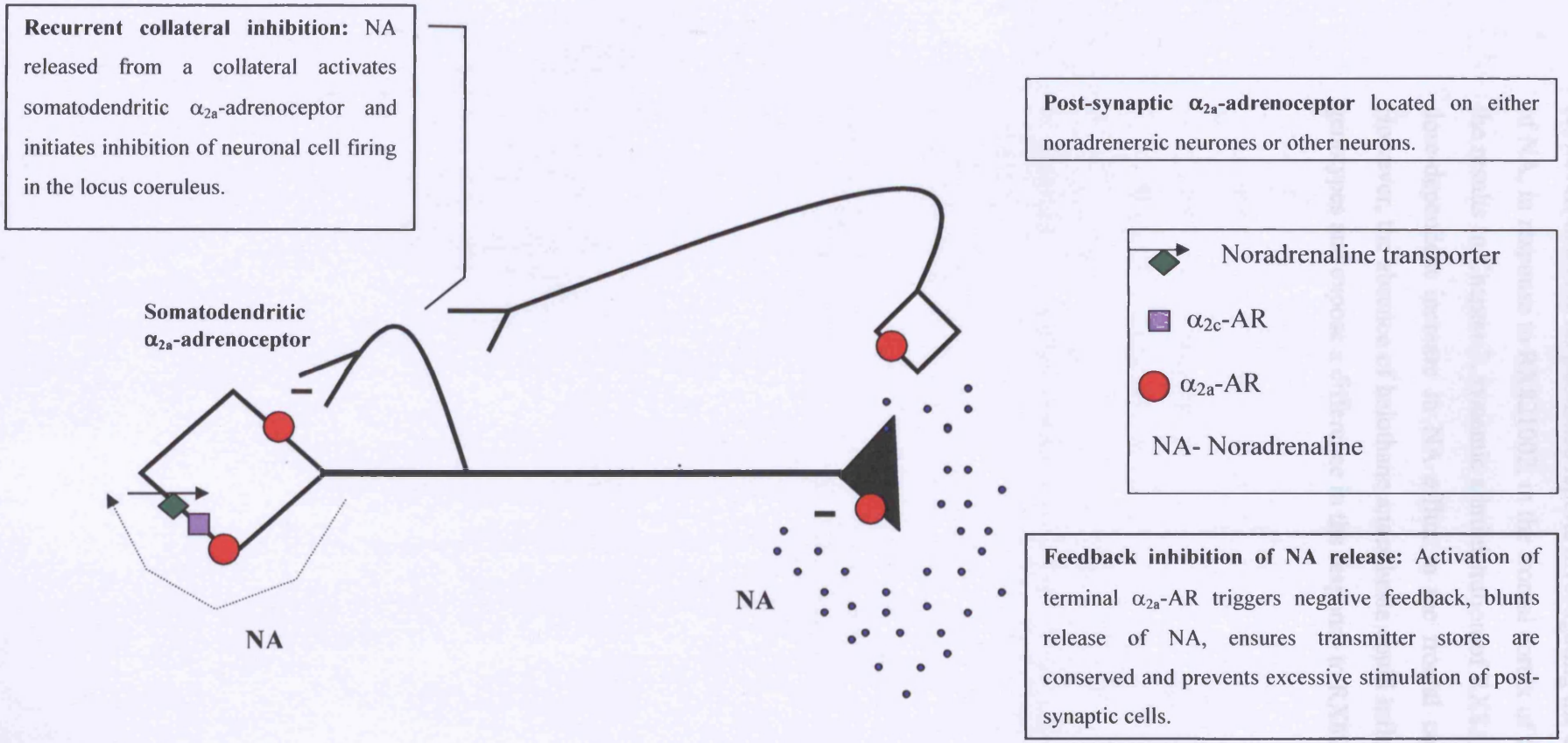


FIGURE 4.1: Regulation of noradrenaline release by the autoregulatory α_2-adrenoceptor, located on cell bodies of LC neurones noradrenergic terminals, and on non-noradrenergic neurones.

However, there are no published reports investigating the electrophysiological or release of NA, in response to RX821002, in the frontal cortex of freely-moving mice. Based on the results in Chapter 3, systemic administration of RX821002 is expected to produce a dose-dependent increase in NA efflux in the frontal cortex of NK1+/+ mice, only. However, the absence of halothane anaesthesia could influence basal NA efflux in both genotypes and expose a difference in the response to RX821002.

4.2 PROTOCOLS

Probes were implanted as described in Section 2.6.2. On the day after surgery, the microdialysis experiment started at 08:00 a.m.

NK1+/+ and NK1-/- mice were divided randomly into 4 groups and given one of the following treatments:

- Saline 10 ml / kg i.p.
- RX821002 0.3 mg / kg i.p.
- RX821002 1 mg / kg i.p.
- RX821002 3 mg / kg i.p.

In each case NA efflux was monitored for 3 h following systemic administration of RX821002.

4.3 RESULTS

4.3.1 Effect of genotype on basal noradrenaline efflux

There was no difference in basal NA efflux pooled across the groups in NK1+/+ (4.72 ± 0.34 fmol / 60 min from T_{-40}-T_0) and NK1-/- mice (5.22 ± 0.36 fmol / 60 min from T_{-40}-T_0).: main effect of 'genotype', ($F_{1,65}$ = 1.13; P = 0.29; T_{-40}-T_0; Figure 4.2a-c). There was no difference between groups of animals destined for different drug treatments (Figure 4.2a-c).

4.3.2 Effect of intraperitoneal injections of saline or RX821002 on noradrenaline efflux in the frontal cortex

4.3.2.1 Injection of saline 10 ml/kg i.p.

A transient increase in NA efflux was apparent after administration of saline 10 ml / kg i.p. in both genotypes. However, this increase was not maintained after the first 20 min post injection and was not different from basal NA efflux (Figure 4.2a-c).

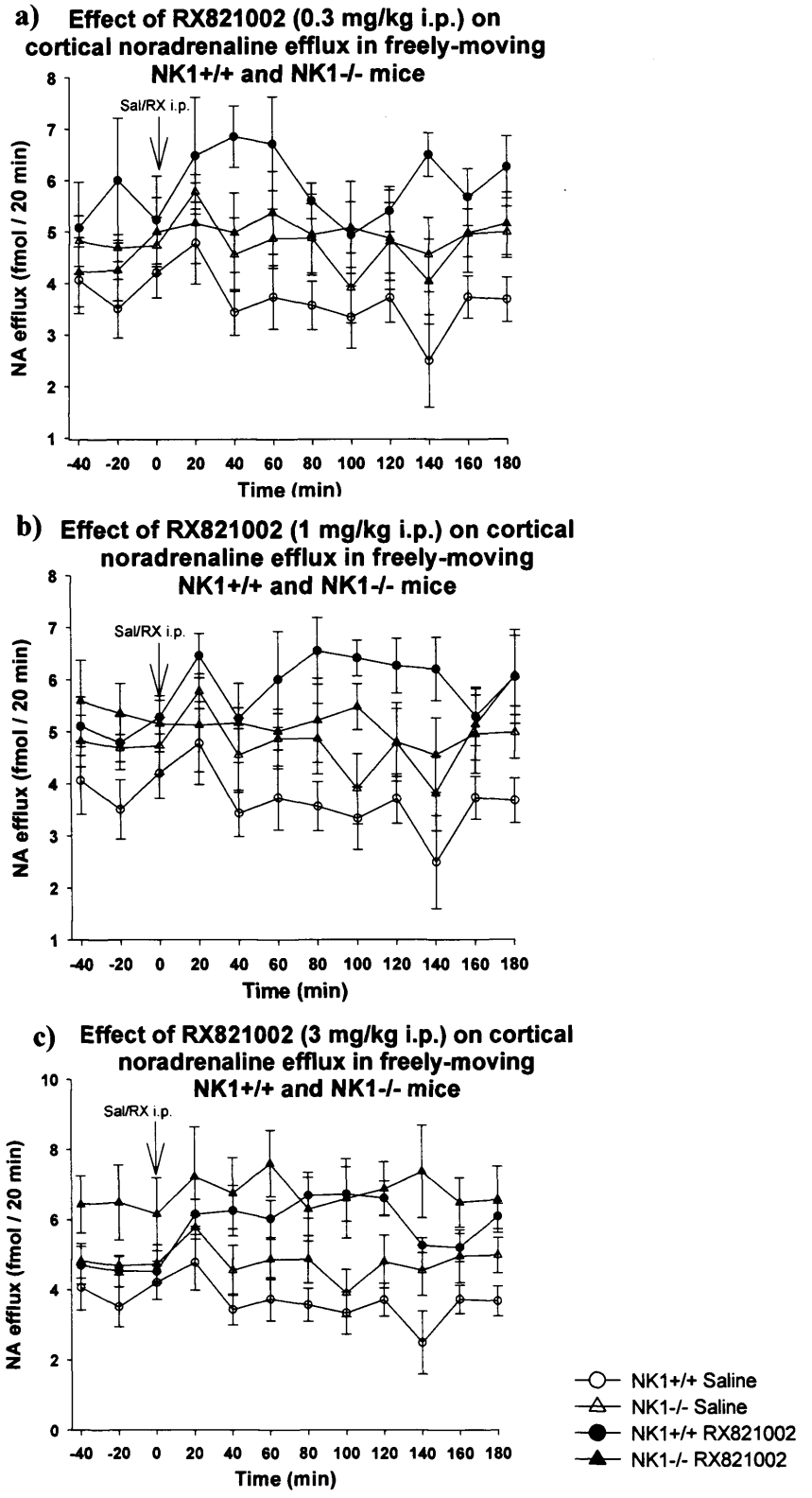


FIGURE 4.2a-c: Raw data. Effect of RX821002 on noradrenaline efflux. RX821002 (0.3 mg / kg i.p.) did not increase noradrenaline efflux in either genotype. At 1 mg / kg i.p. RX821002 increased noradrenaline efflux in NK1+/+ mice, only. At a dose of 3 mg / kg i.p. RX821002 began to produce effects in both genotypes. Values are mean noradrenaline efflux ± s.e.m. n = 9; * $P = \leq 0.05$.

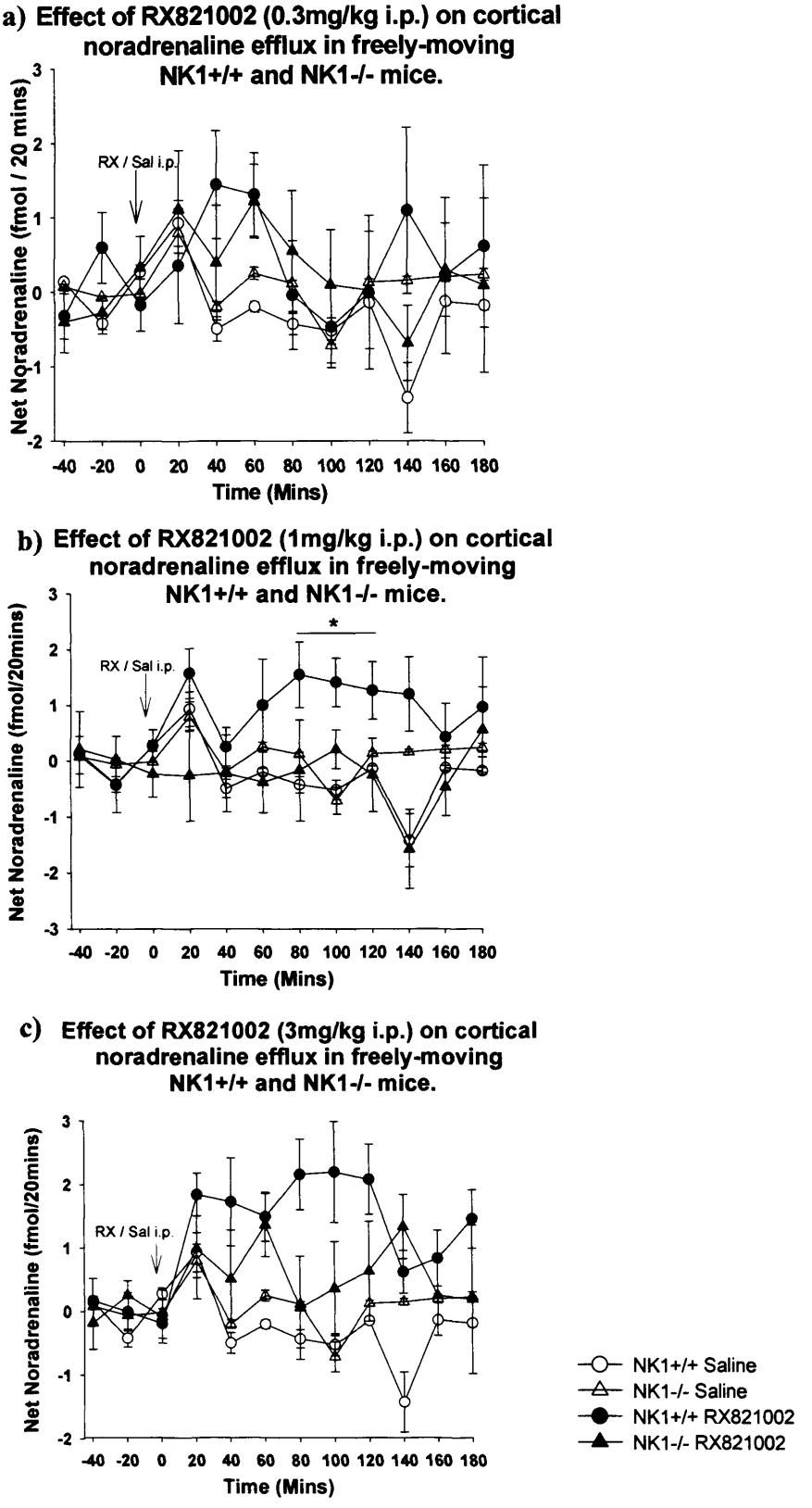


FIGURE 4.3a-c: Effect of RX821002 on net noradrenaline efflux in freely-moving mice. RX821002 (0.3 mg / kg i.p.) did not increase noradrenaline efflux in either genotype. At 1 mg / kg i.p. RX821002 increased noradrenaline efflux in NK1+/+ mice, only. At a dose of 3 mg / kg i.p. RX821002 began to produce effects in both genotypes. Values are mean net noradrenaline efflux ± s.e.m. n = 9; * $P = \leq 0.05$.

4.3.2.2 Injection of RX821002 0.3, 1.0 and 3.0 mg / kg i.p.

Analysis of raw data: Comparison of saline and 0.3 mg / kg i.p. treatment groups revealed a main effect of 'drug' ($F_{1,16} = 7.03$; $P = 0.017$; T_0-T_{180}; Figure 4.2a). Post-hoc analysis showed that RX821002, in NK1+/+ mice, increased NA efflux compared to saline treatment ($P = 0.003$). However, this increase was not different from NK1-/- mice treated with RX821002 (0.3 mg / kg i.p; $P = 0.24$).

Comparison of saline and 1.0 mg / kg i.p. treatment groups revealed a main effect of 'drug' ($F_{1,13} = 6.68$; $P = 0.023$, T_0-T_{180}) and a 'drug x genotype' interaction ($F_{1,13} = 5.17$; $P = 0.041$; Figure 4.2b). From T_{100}-T_{140} a main effect of 'drug' was also found ($F_{1,23} = 8.99$; $P = 0.006$) along with a 'drug x genotype' interaction ($F_{1,23} = 7.26$; $P = 0.013$). Over these three time points RX821002 (1.0 mg / kg i.p.) increased NA efflux in NK1+/+ mice to a greater extent then NK1-/- mice treated with RX821002 (1.0 mg / kg i.p; $P = 0.012$). The RX821002 induced increase in NA efflux was also higher than that observed in saline treated NK1+/+ ($P = 0.001$) and NK1-/- ($P = 0.007$) mice.

Comparison of saline and 3.0 mg / kg i.p. treatment groups revealed a main effect of 'drug' ($F_{1,16} = 9.06$; $P = 0.008$, T_0-T_{180}; Figure 4.2c). However, no 'drug x genotype' interaction was found, suggesting non-specific effects occurring at this higher dose or desensitised α_2-adrenoceptors.

Analysis of net data, revealed the same pattern of results as analysis of raw data (Figure 4.3a-c).

4.4 DISCUSSION

RX821002 crosses the blood-brain barrier (Millan, 1992). Therefore, following systemic administration, the α_2-adrenoceptor antagonist should increase firing-rate of LC noradrenergic neurones (Mateo *et al.*, 1998; Ugedo *et al.*, 1998). It should also block autoreceptors located on noradrenergic nerve terminals and so enhance NA release in terminal projection areas. The increase in NA efflux induced by RX821002 found in these experiments supports this. The 1.5-fold increase above basal levels in NA efflux, seen with RX821002 (1 mg / kg i.p.) in NK1+/+ mice only, is also similar to that observed by (Meana *et al.*, 1997).

4.4.1 Basal noradrenaline efflux is not different in freely-moving NK1+/+ and NK1-/- mice

In contrast to the results presented in Chapter 3 there was no difference in NA efflux between freely-moving NK1+/+ and NK1-/- mice. When mice were placed under halothane-anaesthesia as in Chapter 3, a significant 4-5-fold difference in basal NA efflux was observed between the two genotypes.

It is interesting to note that the average basal NA efflux of NK1+/+ freely-moving mice was higher than halothane-anaesthetised NK1+/+ mice in Chapter 3. These two studies were not randomised together and so direct comparisons are strictly not valid. This is, however, in agreement with studies showing that halothane blocks the response of mesencephalic reticular neurones in the cat (Shimoji *et al.*, 1977). Under halothane anaesthesia LC neurones fire in a slow and regular pattern in the rat (Saunier *et al.*, 1993). Withdrawal from halothane anaesthesia increases the average firing-rate of LC neurones, although NA is normally released during burst-firing. Anaesthetics, such as chloral hydrate and pentobarbital, selectively suppress release, reuptake and metabolism of NA, but have no effect on the basal extracellular concentrations of NA in the medial prefrontal cortex of rats (Pan & Lai, 1995). It was, therefore, predicted that removal of halothane anaesthesia would result in an increase in basal NA efflux in both genotypes.

However, in contrast to NK1+/+ mice, NK1-/- mice demonstrated a reduction in basal NA efflux in the freely-moving situation compared to halothane anaesthesia, suggesting that in NK1-/- mice halothane has an excitatory effect in these experiments.

4.4.2 Effect of RX821002 (0.3 mg / kg) on noradrenaline efflux

In both freely-moving NK1+/+ and NK1-/- mice, 0.3 mg / kg RX821002 did not affect NA efflux. However, under halothane anaesthesia (Chapter 3) a dose of 0.3 mg / kg i.p of RX821002 increased NA efflux approximately 4-fold above basal in NK1+/+ mice, only. Therefore, although there is probably a lower noradrenergic tone at α_2-adrenoceptors under halothane anaesthesia, compared to the freely-moving situation, a lower dose of the α_2-adrenoceptor antagonist produces an increase in NA efflux that is not observed in the freely-moving situation. This could be due to the fact that halothane anaesthesia increases the sensitivity of α_2-adrenoceptors or has a synergistic interaction with α_2-adrenoceptors (Saunier *et al.*, 1993).

4.4.3 NK1-/- mice do not respond to RX821002

In freely-moving NK1-/- mice, NA efflux was unaffected by RX821002 at 0.3 and 1.0 mg / kg i.p., suggesting that the α_2-adrenoceptors in these animals are not functioning efficiently. In contrast, in freely-moving NK1+/+ mice a dose of 1 mg /kg i.p. did increase NA efflux compared to NK1-/- mice. The higher dose of 3 mg / kg i.p. increased NA efflux in both genotypes, perhaps due to non-specific effects, or a lower affinity of α_2-adrenoceptors in NK1-/- mice, perhaps as a result of desensitisation.

4.4.4 Summary and conclusions

An increase in NA efflux in NK1+/+ mice, compared to NK-/- mice, was found with a dose of 1 mg / kg i.p. Freely-moving NK1-/- mice do not respond to RX821002 (0.3 and 1.0 mg / kg i.p.). At the higher dose of 3 mg / kg i.p. the 'drug x genotype' was no longer found suggesting an effect on NA efflux in both genotypes at this dose. Based on the results from this dose-response experiment a dose of 1 mg / kg was chosen for microdialysis studies using the light / dark exploration box (see Chapter 5).

The following chapter describes two complimentary experiments that investigate the behavioural and NA response of NK1+/+ and NK1-/- mice to the non-noxious stressor, novelty. To determine whether any genotype dependent differences in behaviour or NA efflux could be attributed to differences in the α_2-adrenoceptor, NA efflux and behavioural reactions to novelty were monitored following systemic administration of the α_2-adrenoceptor antagonist RX821002 (1 mg/kg i.p.).

CHAPTER 5

EFFECT OF NOVELTY ON BEHAVIOUR AND NORADRENALINE EFFLUX IN NK1+/+ AND NK1-/- MICE

5 EFFECT OF NOVELTY ON BEHAVIOUR AND NORADRENALINE EFFLUX IN NK1+/+ AND NK1-/- MICE

5.1 INTRODUCTION

This chapter presents results from an experiment used to determine the behavioural response of NK1+/+ and NK1-/- mice to a non-noxious naturalistic novel environment, the light dark exploration box (LDEB). *In vivo* microdialysis was also used to determine parallel effects on NA efflux in the prefrontal cortex of NK1+/+ and NK1-/- mice in the same novel environment.

5.1.1 Why use naturalistic behavioural tests?

Physical, aversive stimuli such as restraint (Abercrombie & Jacobs, 1987; Yokoo *et al.*, 1990a; Vahabzadeh & Fillenz, 1994), tail pinch (Aston-Jones & Bloom, 1981b; Vahabzadeh & Fillenz, 1994), and foot-shock (Hirata & Aston-Jones, 1994; Rossetti *et al.*, 1990), increase NA efflux and unit activity of LC noradrenergic neurones. Since these stressors are not commonly experienced by humans, this calls into question their validity when investigating the stress response. Moreover, these laboratory stressors are associated with somatosensory stimulation and cause overt physical discomfort (for review see: (Stanford, 1995)). Whether the changes in the brain produced by these lab stressors are relevant to the effects of non-noxious psychological stimuli is, therefore, debateable (Stanford & Salmon, 1989; Stanford, 1995). Moreover, it is believed that naturalistic stimuli such as 'threat' or 'loss' rather than somatosensory stimuli precipitate the onset of anxiety and depression, respectively in humans (see: (Stanford, 1995)).

Naturalistic stressors, such as exposure to a novel environment might, therefore, be more appropriate for investigating the role of noradrenergic neurones in response to stress.

5.1.2 Response of the locus coeruleus-noradrenergic system to naturalistic stressors

It has been postulated that the LC-noradrenergic system plays an important role as an 'alarm system' (Redmond, Jr. & Huang, 1979). This is supported by the fact that release of NA enhances the reaction of the organism to internal or external stimuli, more specifically the processing of sensory information which accompanies it (Jacobs *et al.*, 1991).

The firing activity of LC neurones in response to novelty has been examined in freely-moving rats (Sara *et al.*, 1994; Vankov *et al.*, 1995). These studies show that LC cells respond in burst to imposed novel sensory stimuli or to novel objects encountered during free exploration. When the stimulus lacks predictive value and no behavioural response is required, the LC response shows rapid habituation (Sara *et al.*, 1994), demonstrating that LC neurones fire in response to the salience of a stimulus. LC cells thus respond to novelty or a change in incoming information, promoting selective attention to the relevant stimuli at the moment of change. Devauges and Sara (1991) using a complex food-motivated maze task, have also observed that the firing-rate of LC neurones is enhanced when an exploring rat encounters a novel object (Devauges & Sara, 1991). Furthermore, the time spent exploring the novel object is increased if the LC is stimulated electrically (Devauges & Sara, 1991) or decreased if NA release is inhibited by an α_2-adrenoceptor agonist, such as clonidine (Sara *et al.*, 1995).

NA released in target forebrain areas acts to sharpen or direct attention to relevant stimuli, thereby 'alerting' the animal and orienting them towards the stimuli (Aston-Jones & Bloom, 1981b; Foote *et al.*, 1991). Supporting the role played by the LC in regulating attentional state or vigilance (Aston-Jones *et al.*, 1991a).

Strong evidence is available to support an increase in activation of LC neurones in response to the psychologically aversive features of a stimulus (Tanaka *et al.*, 1991b), further supporting the use of non-noxious naturalistic stressors, over those that posses a somatosensory component in their response. However, the exact role played by NA in response to adverse stressful effects is still unclear. It is possible that NA release follows a bell-shaped dose response curve (Stanford, 1995), and that the increase in NA

in response to stressful stimuli represents a coping strategy, too much NA release, and the response can become detrimental to the animal.

Nevertheless, naturalistic stimuli, which do not result in physical discomfort, increase the firing activity of LC neurones. This supports the use of the non-noxious novel environment of the LDEB to investigate the stress response of the noradrenergic system in NK1+/+ and NK1-/- mice. We predict that noradrenergic neurones of NK1+/+ and NK1-/- mice will respond differently to the novel environment presented by the LDEB.

5.1.3 Behavioural response of NK1+/+ and NK1-/- mice in stress-inducing tests

The behavioural phenotypes of NK1+/+ and NK1-/- mice have been tested in various stress paradigms: e.g. resident-intruder, separation-induced vocalisation, novelty-suppressed feeding, forced swim test and the tail suspension test. The results from these tests lead to the conclusion that the phenotype of the NK1-/- mice resembles that of the NK1+/+ counterpart treated with an established anxiolytic / antidepressant compound e.g. fluoxetine (Rupniak *et al.*, 2001). However, the behaviours exhibited in NK1-/- mice in the open-field and elevated-plus maze are inconsistent. No difference between genotypes in the open field and elevated-plus maze was found by De Felipe *et al.*, (1998) and Rupniak *et al.*, (2001), whereas an increase in exploratory behaviour in the open field and a reduction in stress related responses in the elevated plus-maze has been found by (Santarelli *et al.*, 2001; Santarelli *et al.*, 2002).

Behaviour produced in the LDEB is strongly strain dependent (Rodgers *et al.*, 2002). 129/SvEv strains of mice demonstrate higher levels of anxiety-like behaviour (conventional and / or ethologically) (Rodgers *et al.*, 2002). The studies conducted by Santarelli *et al.*, (2001) were performed on NK1-/- mutants from a pure 129/SvEv background (Santarelli *et al.*, 2001). Therefore, genetic differences between the NK1-/- mice developed from a pure background strain of 129/SvEv mice and those developed from a hybrid line (129/Sv X C57BL/6) may account for the differences observed between the two groups.

The mice used in these LDEB studies are derived from C57BL/6 blastocysts implanted with genetically manipulated 129SvEv stem cells (De Felipe *et al.*, 1998) which were

crossed onto an MF1 background. The behaviour of NK1-/- mice bred from this strain is not different from that of their NK1+/+ counterpart in either the open-field or elevated-plus maze (Rupniak *et al.*, 2001; Murtra *et al.*, 2000; David *et al.*, 2004), however, there are marked genotype dependent behavioural differences between NK1+/+ and NK1-/- mice in the LDEB (Herpfer *et al.*, 2005), which may be attributed to differences in the regulation of the noradrenergic nervous system between these two genotypes.

The LDEB has the additional advantage that it can be performed in combination with *in vivo* microdialysis. Therefore, changes in behaviour observed in the LDEB can be related to changes in NA efflux of NK1+/+ and NK1-/- mice.

5.1.4 Microdialysis studies of the noradrenergic response to naturalistic stimuli

Measurement of the NA metabolite 3-methoxy-4-hydroxyphenylethlene glycol (MHPG) provides a marker for a decrease or increase in NA release. Accumulation of MHPG is observed in the brains of rats watching conspecifics experiencing foot-shock (Iimori *et al.*, 1982; Tanaka *et al.*, 1991a). The increase in MHPG produced by this psychological stressor is localised to limbic regions such as the amygdala, LC and hypothalamus. Whereas the changes produced by laboratory stressors appear more widespread throughout the brain (Iimori *et al.*, 1982).

Return of rats to an environment in which they previously experienced footshock, tests emotional stress without the physical component. Microdialysis studies have also demonstrated an increase in NA efflux in the hypothalamus of rats when they are returned to the environment in which they experienced footshock (Yokoo *et al.*, 1990b).

Our lab has previously used the LDEB as a naturalistic stressor in a number of microdialysis studies. These studies found that different naturalistic stimuli, result in incremental changes in NA efflux, in the frontal cortex (Dalley & Stanford, 1995). An increase in cortical (Dalley & Stanford, 1995) and hypothalamic (McQuade *et al.*, 1999) NA efflux, was observed, when rats were transferred to the novel, brightly lit environment of the LDEB, plus or minus an unfamiliar conspecific. A conditioned stimulus (tone), predicting transfer of a rat to the same novel aversive environment, also

caused an increase in cortical but not hypothalamic NA efflux (McQuade & Stanford, 2000).

Firing activity and NA efflux of LC and hippocampal noradrenergic neurones is, therefore, increased in response to novel and salient external stimuli or a change in incoming information (Simson & Weiss, 1989; Aston-Jones *et al.*, 1991a; Sara *et al.*, 1994; Dalley *et al.*, 1996; Kitchigina *et al.*, 1997; McQuade *et al.*, 1999). Strong evidence supports the use of non-noxious naturalistic stimuli e.g. the LDEB, to investigate the noradrenergic nervous system in the behavioural response / emotional impact of a naturalistic stressor.

5.1.5 Anxiety-like behaviours and the light dark exploration box

It must be emphasised that the LDEB is typically used as a model to screen anxiolytic drugs and so, by implication, reflects anxiety-like behaviour. However, for these studies it was used purely to profile the behavioural response to novelty, in such a way that genotype-dependent behavioural differences could be attributed to differences in the regulation of the noradrenergic system between NK1+/+ and NK1-/- mice. The effects of typical anxiolytics such as benzodiazepines (BDZs) were not used to validate the anxiety-like behavioural response in this study.

In the prototypical light / dark shuttle box (behaviour only monitored for 3 min), BDZs increase exploratory behaviour. This is measured as an increase in transitions between the light and dark compartments (Crawley & Goodwin, 1980). Costall *et al.,* (1989) found that an increase in exploratory behaviour was associated with an increase in the time spent in the light compartment and parallels an increased duration in the light zone and an increase in transitions (Costall *et al.*, 1989). Rearing activity of mice is also believed to be an indicator of exploratory behaviour (Rodgers *et al.*, 2002).

Evidence suggests that the novelty of the white compartment initiates a number of avoidance behaviours in mice. Stretched-attend postures, where the mouse stretches forward and retreats to original position, and flat back approach, (classically known as risk-assessment behaviours), are believed to represent active avoidance responses, as is leaving the light compartment (Hascoet *et al.*, 2001).

A parameter that influences exploratory behaviour and, therefore, an important variable to control for, is age and neuronal maturation of the animals. Hascoet *et al,* (1999) have shown that the optimum age of mice in the LDEB test is 4 weeks. At this age mice spend 58 % of the total duration in the dark compartment. Older mice (8 weeks old) exhibit an increase in total activity, characterised by an increase in movements in each compartment, together with an increase in the number of transitions (Hascoet *et al.*, 1999). Therefore, mice in these present experiments were used at the age of 4-6 weeks (25-30g). Other variables that can influence experimental outcome include prior test experience (Holmes *et al.*, 2001) and time of day when experiment is performed (Costall *et al.*, 1989). All tests were, therefore, carried out on naive mice at the same time of day.

Since previous microdialysis studies have demonstrated an increase in NA efflux in rats when they are placed in the novel environment of the LDEB, it is likely that the novel environment, provided by the LDEB, would increase the firing activity of LC neurones in NK1+/+ and NK1-/- mice, and possibly increase NA efflux. These current studies are, therefore, particularly concerned with determining behavioural differences between NK1+/+ and NK1-/- mice in the LDEB, an environment which they may find stressful / aversive. Having determined any genotype dependent behavioural differences further interest lies in whether these behaviours can be attributed to a difference in the noradrenergic system of these mice. To investigate differences in the noradrenergic system, of NK1+/+ and NK1-/- mice, these studies also focused on the behavioural response to novelty following challenge with compounds known to modify NA, most notably α_2-adrenoceptor antagonists.

5.1.6 Effect of α_2-adrenoceptor antagonists on behaviour

Several studies have drawn parallels between activation of LC noradrenergic neurones and behavioural arousal (Carli *et al.*, 1983; Cole & Robbins, 1992; Robbins, 1997).

It has been postulated that a novel environment causes an increase in NA which is further potentiated by systemic administration of an α_2-adrenoceptor antagonist e.g. idazoxan (Devauges & Sara, 1990) eliciting an increase in the duration spent investigating novel objects.

Conversely it has been reported that administration of idazoxan decreases exploratory behaviour in a novel environment in a multicompartment chamber (Berridge and Dunn, 1987). Similarly, (Haapalinna *et al.*, 1999) have found a decrease in the exploratory behaviour of rats dosed with the α_2-adrenoceptor antagonists yohimbine or atipamezole in the staircase test. This group also report that atipamezole has no effect on spontaneous motor activity in the staircase test (Haapalinna *et al.*, 1999).

These conflicting results demonstrate that an increase in NA release, following antagonism of α_2-adrenoceptors, does not always result in an increase in exploratory or locomotor behaviour. Furthermore, they demonstrate that α_2-adrenoceptor antagonists do have different effects on behavioural response to novelty but these effects are as yet unclear and sometimes conflicting.

5.1.7 Aims

Although evidence is available documenting the effect of novel naturalistic stimuli on behaviour and NA efflux in rats, there are no studies documenting this response in mice. These studies are based on the hypothesis that the higher NA efflux in NK1-/- mice may be a result of the α_2-adrenoceptor malfunctioning in some way. It was, therefore, predicted that the difference in basal NA efflux between the two genotypes would result in differences in the exploratory behaviour in a novel environment in the LDEB.

Systemic administration of the α_2-adrenoceptor antagonist, RX821002, was used to investigate whether antagonism of the α_2-adrenoceptor modifies this behavioural response to novelty, and whether this differs between genotypes.

Subsequent studies, focused on the effect placement in the LDEB has on NA efflux, in NK1+/+ and NK1-/- mice, using *in vivo* microdialysis. Systemic administration of RX821002 was again used to determine whether antagonism of the α_2-adrenoceptor effects NA efflux when mice are placed in a novel environment, and whether this is different between NK1+/+ and NK1-/- mice.

5.2 PROTOCOLS

5.2.1 Behavioural light dark exploration box protocol

Experiments were conducted between 13:00-15:00 and 15:30-17:30 p.m. every day. Two LDEB were used side by side, so that animals were run in pairs. Animals were placed in the neutral zone (2 lux) to habituate for 60 min. This 60 min habituation period was chosen to allow time for efflux to stabilise when the LDEB protocol was combined with *in vivo* microdialysis. At 14:00 p.m. mice were dosed with either saline 10 ml / kg i.p. or RX821002 (0.3 mg / kg i.p.) and left in the dark side of the exploration box for a further 30 min. The mice were then lifted out from the dark zone and placed in the centre of the light novel compartment (10 lux) facing away from the guillotine style door, which was raised to allow animals to traverse freely between the two compartments. Behaviour was then monitored for a further 30 min using a Sony Handycam Vision video recorder (see Section 2.7.1.2 for behaviours scored). At the end of the experiment the animals were removed from the LDEB and killed using an overdose of CO_2 and cervical dislocation. The LDEB was then cleaned thoroughly with alcohol and water. The experiment was repeated at 15:30 p.m. (see Figure 5.1 for protocol).

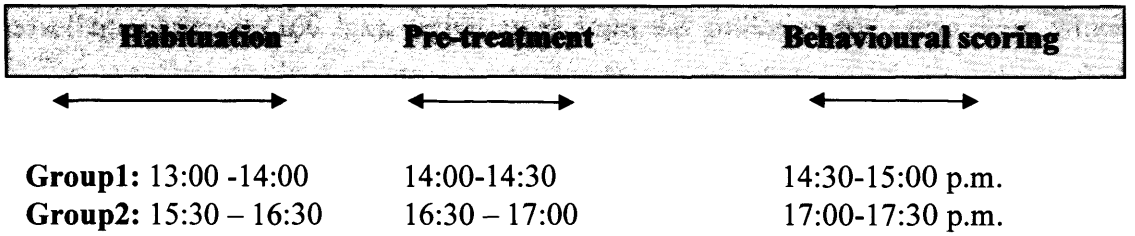


FIGURE 5.1: Experimental protocol for behavioural testing using the light / dark exploration box.

5.2.2 Microdialysis in the light dark exploration box protocol

Probes were implanted as described in Section 2.6.2.

Due to diurnal variation in NA efflux and the behavioural dependence of the LDEB on the time of day (Costall *et al.*, 1989), the timing of the behavioural experiment in the LDEB was synchronised with harvesting of extracellular NA during *in vivo* microdialysis. However, due to the necessary probe stabilisation, and the time required to obtain three consecutive stable basal samples, it was decided to bring the experiment forward 60 min. Animals were removed from their homecage, where they had been left overnight after probe implantation, and placed in the neutral zone (6 lux) for 60 min to allow for probe equilibration. Following equilibration, samples were collected every 20 min until 3 consecutive stable samples established a stable baseline (typically 60 min). Mice were then dosed with either saline (10 ml / kg i.p.) or RX821002 (1 mg / kg i.p.) and placed back in the neutral zone. This dose of RX821002 was chosen based on the results from Chapter 4. A dose of 1 mg / kg i.p. produced a significant increase in NA efflux compared to saline treated NK1+/+ mice, in their home cage, and avoided possible adverse effects that may have been encountered with a higher dose. A 10-min sample was then collected, followed by a 20-min sample to allow for the 30-min pre-treatment time. Following pre-treatment, mice were placed in the centre of the light novel compartment (160 lux), facing away from the guillotine style trap door, which remained in place throughout the experiment. The counter-balanced lever arm attached to the swivel was secured to an upright stand attached to the side of the light zone. NA efflux was monitored for a further 120 min. (see Figure 5.2 for protocol)

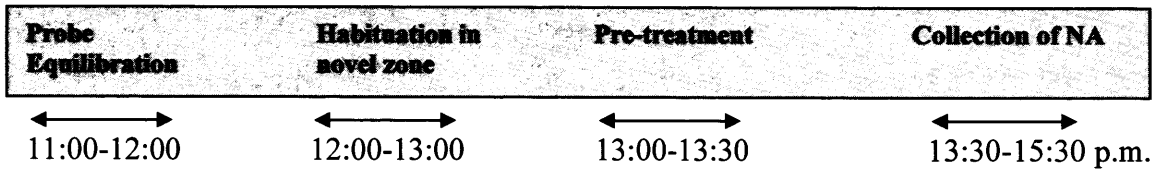


FIGURE 5.2: Experimental protocol for *in vivo* microdialysis in the light / dark exploration box.

5.2.3 Data analysis

Behavioural data were analysed using two-way ANOVA with Levene's test for normal distribution, followed by one-way ANOVA to reveal differences between groups. If the Levene's test was significant, data were analysed using Kruskull Wallis non-parametric ANOVA, followed by Mann-Whitney U (non-parametric) analysis. Co-variate analysis was also performed using total locomotor activity as a covariate, to identify differences that may have been masked or exaggerated due to a higher level of total spontaneous locomotor activity in one of the genotypes.

For microdialysis data the NA content is expressed as fmol / 20 min without correction for probe recovery. When investigating the effect of the novel environment on NA efflux, the net change in NA efflux was calculated in order to compensate for any differences in basal NA across subjects. This involved subtracting the average of the last three basal samples, taken whilst the mouse was in the neutral compartment, from all samples (Dalley *et al.*, 1996). Both absolute and net microdialysis data were analysed using repeated measures ANOVA with 'bin' and 'time' as 'within subjects' factors and 'genotype' and 'drug' treatment as 'between subjects' factors. If data violated the Mauchley's test for sphericity, then the Greenhouse-Geisser 'ε' correction factor was used. If ANOVA revealed a main effect of 'bin','drug', 'genotype' or a 'drug x genotype' interaction, the data were further analysed using a post-hoc one-way ANOVA or least significant difference (LSD) to identify significant differences between groups or pairs of data. The criterion for statistical significance was set at $\boldsymbol{P \leq 0.05}$.

5.3 RESULTS

5.3.1 Behavioural response to the light / dark exploration box in NK1+/+ and NK1-/- mice

5.3.1.1 Effect of NK1 receptor disruption on behaviour

Of the 12 behaviours scored, 5 behavioural responses differed between the two genotypes (Figure 5.3; Table 5.1).

- ***Total activity*** in both the light and dark zones of the shuttle box was higher in NK1-/- mice ($F_{1,27} = 5.13$; $P = 0.03$).
- ***Total number of rears*** were higher in NK1-/- mice ($F_{1,26} = 5.49$; $P = 0.028$).
- ***Total time spent in the light*** zone was lower in NK1-/- mice ($F_{1,26} = 4.97$; $P = 0.036$).

Of particular interest was the observation that NK1-/- mice perform fewer, and spend less time in, risk assessment behaviours:

- NK1-/- mice spent less time in ***flat-back approach*** ($F_{1,25} = 12.4$; $P = 0.002$) and,
- performed fewer ***stretched-attend*** *postures*, ($F_{1,25} = 49.5$; $P < 0.001$).

To determine if any genotype-dependent behaviours were due to differences in locomotor behaviour, observed between NK1+/+ and NK1-/- mice, this was treated as a covariate in ANCOVA. After ANCOVA, three behaviours still differed between the genotypes. However, the apparent difference in total rearing behaviour was solely because of their difference in locomotor activity.

- ***Time in the light zone*** was less in NK1-/- mice ($F_{1,26} = 8.6$; $P = 0.008$).
- ***Time spent in flat-back approach*** and ***number of stretched-attend postures*** were less in NK1-/- mice ($F_{1,25} = 7.29$; $P = 0.013$ and $F_{1,25} = 39.58$; $P = 0.001$) respectively.

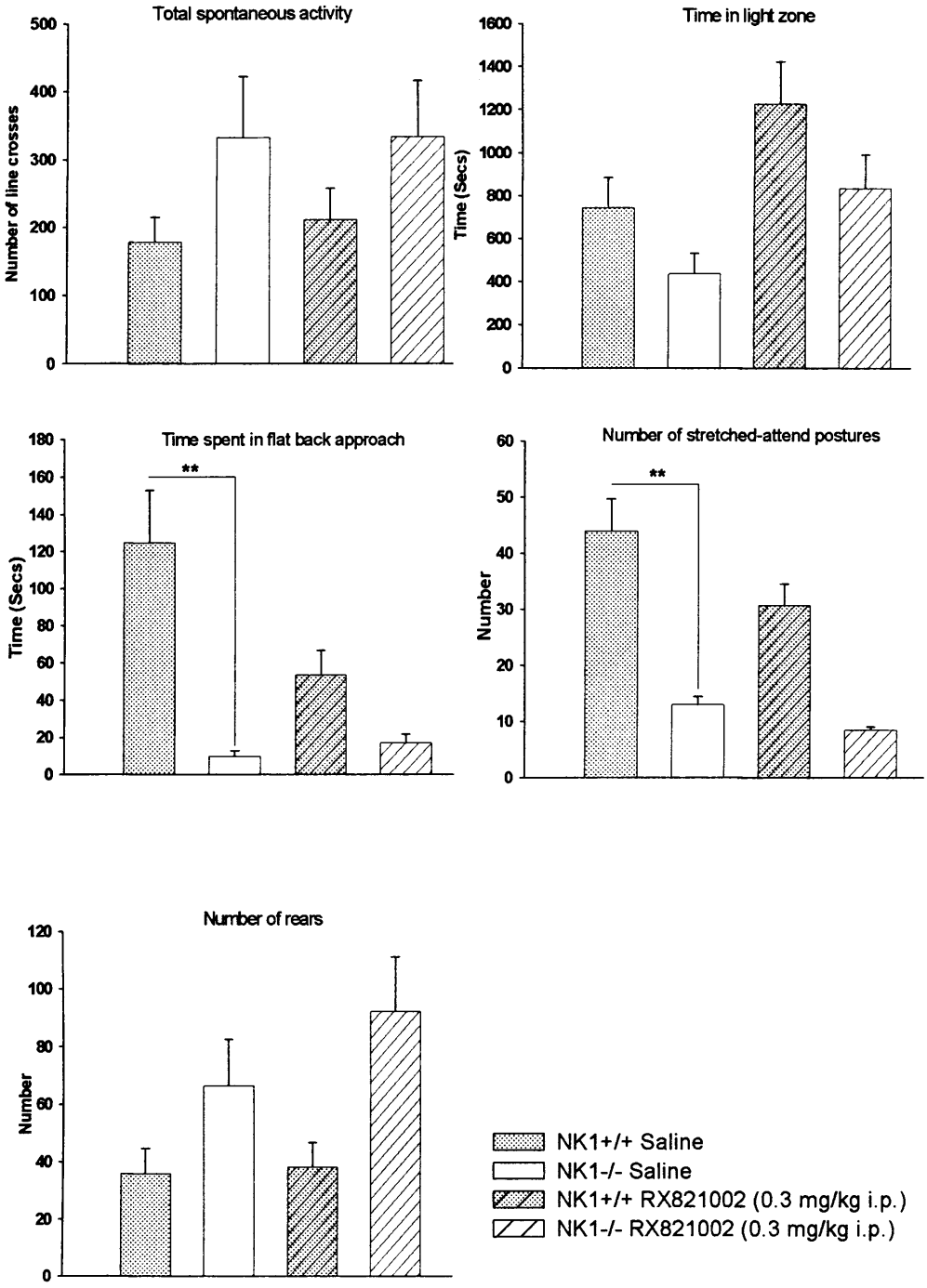


FIGURE 5.3: Behaviours that show a main effect of 'genotype' in the light / dark exploration box. Asterisks indicate differences between individual treatment groups. For statistical details see Table 5.1. **$P < 0.001$. Values are mean ± s.em. n = 7.

Behaviour	Two-way ANOVA	NK1+/+ Vehicle	NK1-/- Vehicle	NK1+/+ RX821002	NK1-/- RX821002
Main effect of genotype					
Number of Stretched-attend postures	Before ANCOVA gg After ANCOVA gg	44 ± 5.7	13 ± 1.4	30.7 ± 3.8	8.5 ± 0.6
Flat back approach (S)	Before ANCOVA gg After ANCOVA gg	124.8 ± 28.1	9.9 ± 3.1	53.4 ± 13.29	17 ± 4.8
Total time in light (S)	Before ANCOVA g After ANCOVA gg	742.3 ± 142.2	435.5 ± 95.8	1226.25 ± 196.8	833.1 ± 156.5
Number of rears	Before ANCOVA gg After ANCOVA no significance	36 ± 8.6	66.5 ± 16.1	38.3 ± 8.4	92.3 ± 18.9
Locomotor activity	g	223.4 ± 41.8	406.9 ± 107.5	235.4 ± 45	436.4 ± 99.8

TABLE 5.1: behaviours demonstrating a main effect of genotype in the light / dark shuttle box. Values are mean ± s.e.m. n = 7. Two-way ANOVA: main effect of 'genotype': 'g' $P \leq 0.05$, 'gg' $P < 0.01$.

5.3.1.2 Behaviours affected by α_2-adrenoceptor antagonism independent of genotype

A number of behaviours were altered by RX821002, irrespective of genotype (Figure 5.4; Table 5.2).

- ***Total number of grooms*** ($F_{1,26} = 5.72$; $P = 0.025$ 'drug')
- ***Total time spent grooming*** ($F_{1,25} = 12.79$; $P = 0.002$; 'drug')
- ***Time spent in the light zone*** ($F_{1,26} = 7.89$; $P = 0.01$; 'drug')

were all increased in both genotypes.

- ***Stretched-attend postures*** were reduced in NK1+/+ and NK1-/- mice, before ANCOVA ($F_{1,25} = 5.53$; $P = 0.028$; 'drug').
- ***Flat back approach*** appeared reduced in NK1+/+ mice and appeared increased in NK1-/- mice, ($F_{1,25} = -4.92$; $P = 0.04$; 'drug') but no 'drug x genotype' interaction was evident.

After ANCOVA the same effects were observed.

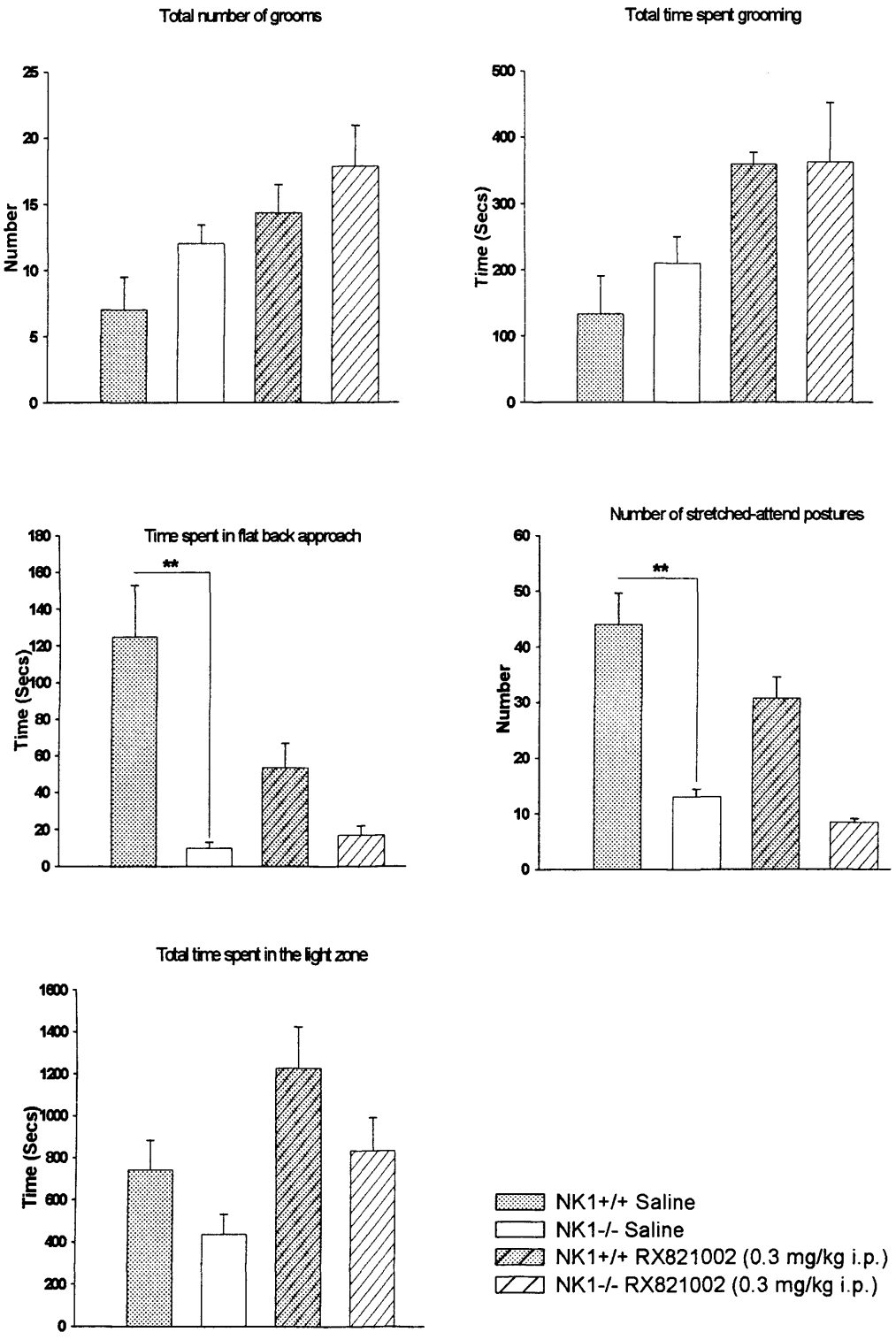


FIGURE 5.4: Behaviours that show an overall effect of 'drug' in the light / dark exploration box. Asterisks indicate differences between individual treatment groups. For statistical details see Table 5.2. ** $P < 0.001$. Values are mean ± s.e.m. n = 7.

Behaviour	Two-way ANOVA	NK1+/+ Vehicle	NK1-/- Vehicle	NK1+/+ RX821002	NK1-/- RX821002
Main effect of drug					
Total time grooming (S)	Before ANCOVA dd After ANCOVA dd	133.6 ± 57.1	210.2 ± 40.1	359.6 ± 17.3	363 ± 89.6
Total number of grooms	Before ANCOVA d After ANCOVA d	7 ± 2.5	12 ± 1.4	14.3 ± 2.1	17.9 ± 3.1
Total time in light (S)	Before ANCOVA dd After ANCOVA d	742.3 ± 142.2	435.5 ± 95.8	1226.3 ± 196.8	833.1 ± 156.5
Number of Stretched-attend postures	Before ANCOVA d After ANCOVA d	44 ± 5.7	13 ± 1.4	30.7 ± 3.8	8.5 ± 0.6
Time spent in flat back approach (S)	Before ANCOVA d After ANCOVA d	124.8 ± 28.1	9.9 ± 3.1	53.4 ± 13.29	17 ± 4.8

TABLE 5.2: Behaviours demonstrating a main effect of drug in the light / dark exploration box. Values show mean ± s.e.m. Two-way ANOVA: main effect of 'drug': 'd' $P \leq 0.05$, 'dd' $P < 0.01$.

5.3.1.3 Behavioural effects of α_2-adrenoceptor antagonism that depend on genotype

Systemic administration of the α_2-adrenoceptor antagonist RX821002 (0.3 mg / kg i.p.) produced a number of behavioural changes in response to novelty that were observed in NK1+/+ mice only (Figure 5.5; Table 5.3).

- ***Time spent grooming in the light zone*** was increased in NK1+/+ mice only. ($F_{1,25} = 8.1$; $P = 0.009$; 'drug x genotype' interaction)
- ***Time to return to the light zone*** was significantly reduced in NK1+/+ mice only ($F_{1,24} = 4.55$; $P = 0.045$; 'drug x genotype' interaction).

Although RX821002 had no significant effect on locomotor activity, again, locomotor activity was chosen as the covariate for ANCOVA, to determine if any of the apparent 'drug x genotype' interactions observed could be explained by genotype differences in locomotor activity.

After ANCOVA:

- ***Number of rears in the dark*** was reduced in NK1+/+ mice only ($F_{1,27} = 5.83$; $P = 0.024$; 'drug x genotype' interaction).
- ***Number of returns to the light zone*** was reduced in NK1+/+ mice only ($F_{1,26} = 8.45$; $P = 0.008$; 'drug x genotype' interaction).
- ***Latency to leave the light zone*** was increased in NK1+/+ mice only ($F_{1,24} = 5.55$; $P = 0.03$; 'drug x genotype' interaction).

This indicates that the greater locomotor activity of NK1-/- mice, was masking 'drug x genotype' interaction. A significant 'drug x genotype' interaction was still present in ***time spent grooming in the light*** ($F_{1,25} = 7.63$; $P = 0.012$) and ***time to return to the light zone*** ($F_{1,24} = 4.36$; $P = 0.05$), suggesting that the difference in locomotor activity between NK1+/+ and NK1-/- mice did not affect these behaviours altered by α_2-adrenoceptor antagonism in NK1+/+ mice.

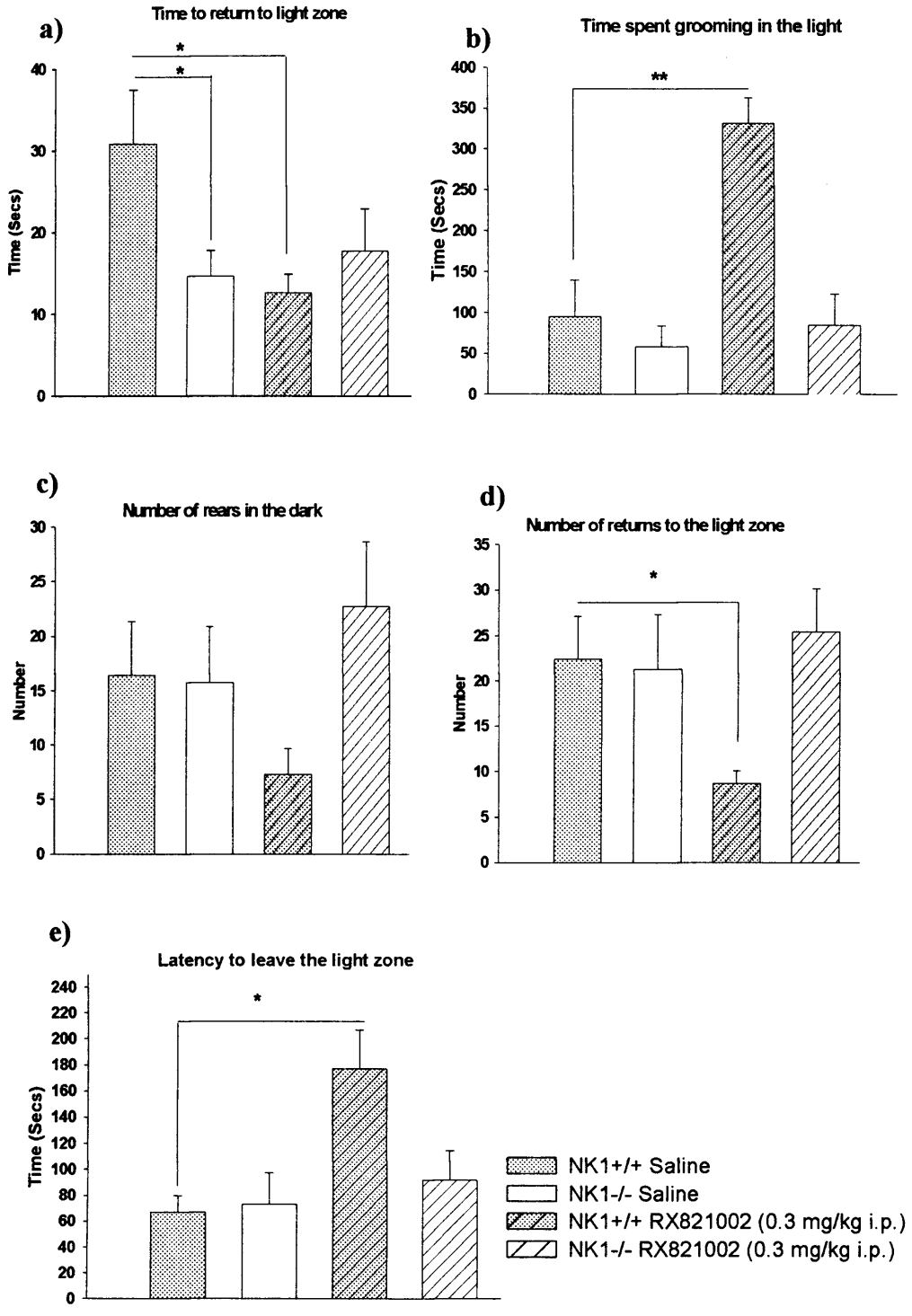


FIGURE 5.5: Behaviours that show a 'dose x genotype' interaction in the light / dark exploration box. Asterisks indicate differences between individual treatment groups. *$P < 0.05$; **$P< 0.01$. a) and b) significance before and after ANCOVA. c), d) and e) significance only after ANCOVA. Values are mean ± s.e.m. n = 7. For statistical details see Table 5.3.

Behaviour	Two-way ANOVA	NK1+/+ Vehicle	NK1-/- Vehicle	NK1+/+ RX821002	NK1-/- RX821002
Drug x Genotype interaction					
Number of returns	Before ANCOVA nothing After ANCOVA ii	22.43 ± 4.7	21.3 ± 6	8.7 ± 1.4	25.4 ± 4.8
Time grooming in the light (S)	Before ANCOVA dd gg ii After ANCOVA dd gg ii	94.8 ± 44.6	58.4 ± 25.7	332 ± 31.2	84.6 ± 38.1
Latency to leave light zone (S)	Before ANCOVA nothing After ANCOVA dd i	67 ± 12.6	73.2 ± 24.4	177.1 ± 29.9	91.8 ± 22.6
Number of rears in the dark	Before ANCOVA nothing After ANCOVA i	16.4 ± 4.9	15.7 ± 5.2	7.3 ± 2.3	22.7 ± 5.9
Time to return to light zone (S)	Before ANCOVA i After ANCOVA i	30.9 ± 6.6	14.6 ± 3.2	12.6 ± 2.2	17.7 ± 5.2

TABLE 5.3: Behaviours which show a drug x genotype interaction in the light / dark shuttle box. Values are mean ± s.e.m. n = 7. Two-way ANOVA: main effect of 'drug': 'dd' $P < 0.01$; main effect of 'genotype': 'gg' $P < 0.01$; 'drug x genotype' interaction: 'i' ≤ 0.05; 'ii' $P < 0.01$.

5.3.2 *In vivo* microdialysis in the light / dark exploration box

5.3.2.1 *Basal noradrenaline efflux between genotypes*

No difference between basal NA efflux was found between NK1+/+ and NK1-/- mice ($F_{1,39} = 2.36$; $P = 0.13$; T_{-40}-T_0;'genotype'; Figure 5.6a). Average basal NA efflux was 9.88 ± 2.06 fmol / 20 min in NK1+/+ mice and 6.77 ± 1.23 fmol / 20 min in NK1-/- mice. There was also no difference between groups destined for different drug treatments ($F_{1,39} = 1.56$; $P = 0.22$; 'drug'; Figure 5.6a).

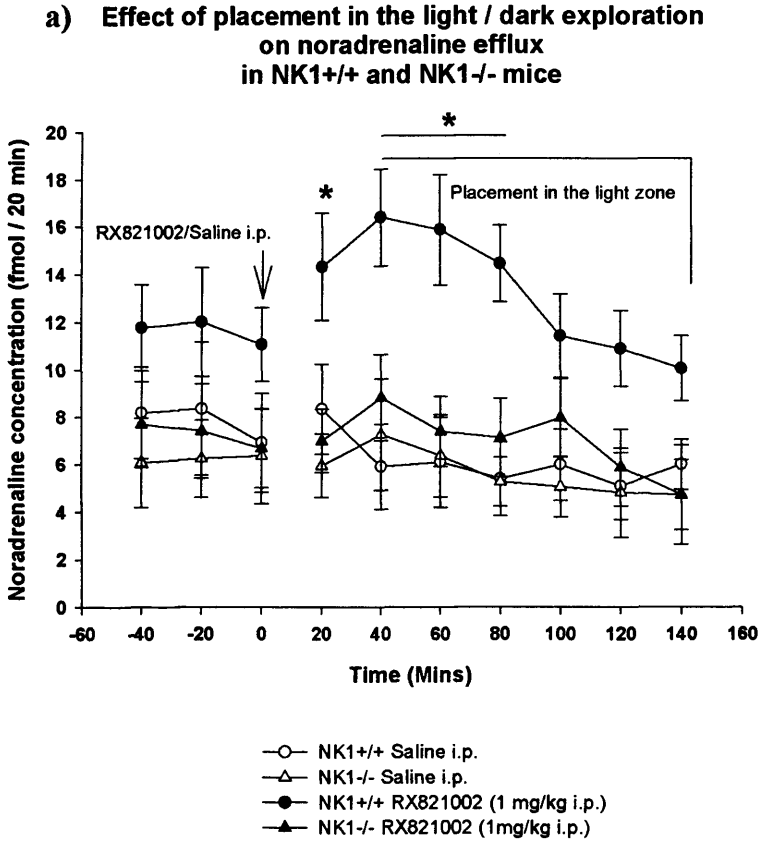
5.3.2.2 *Effect of saline or RX821002 injection on noradrenaline efflux in NK1+/+ and NK1-/- mice*

Raw data: Before placement in the novel zone, at time T_{20}, RX821002 (1mg / kg i.p.) increased NA efflux in NK1+/+ mice, only (Figure 5.6a). This increase in NA efflux was greater than in NK1-/- mice treated with RX821002 ($P = 0.01$) and greater than both saline treated NK1+/+ ($P = 0.025$) and NK1-/- ($P = 0.004$) mice. Saline injection did not increase NA efflux in either genotype (Figure 5.6a).

5.3.2.3 *Effect of placement in the novel light zone on noradrenaline efflux*

Raw data: From T_{40}-T_{80} a main effect of 'drug' was found ($F_{1,35} = 9.15$; $P = 0.005$), and a 'drug x genotype' interaction ($F_{1,35} = 4.73$; $P = 0.037$). Post-hoc analysis revealed that RX821002 increased NA efflux in NK1+/+ mice, only, and only for the first 60 min after placement in the light novel compartment. This increase in NA efflux in RX821002 treated mice was greater than NK1-/- mice treated with RX821002 ($P = 0.006$). The increase in NA efflux in RX821002 pre-treated NK1+/+ mice was also greater than saline pre-treated NK1+/+ ($P = 0.001$) and NK1-/- ($P = 0.001$) mice (Figure 5.6a).

Net data: Analysis of the net increase in NA efflux did not reveal a difference between groups (Figure 5.6b). From T_{40}-T_{80} the main effect of 'drug' was approaching significance ($F_{1,35}$; $P = 0.059$). However, the 'genotype x drug' interaction was not significantly different ($F_{1,35} = 3.11$; $P = 0.087$).



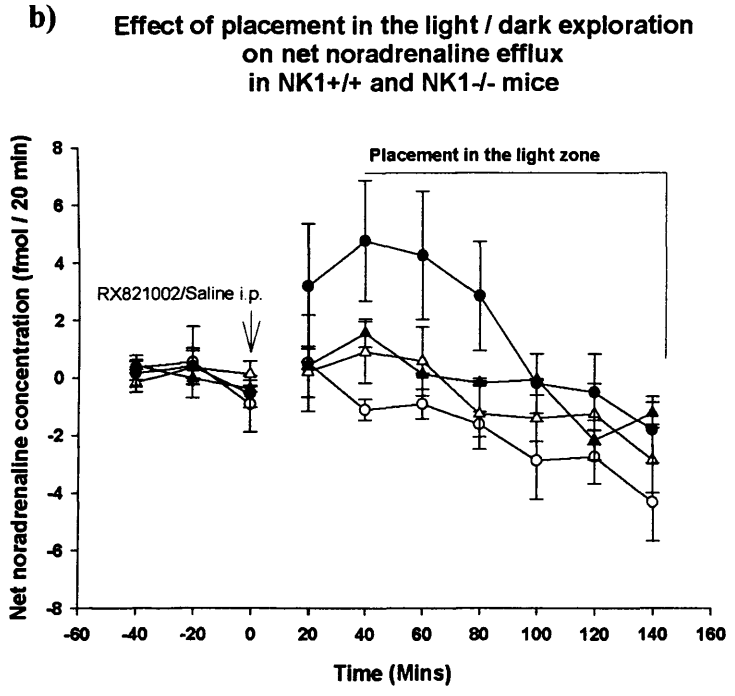


FIGURE 5.6: Effect of placement in the light dark / exploration box on a) raw noradrenaline efflux and b) net noradrenaline efflux in NK1+/+ and NK1-/- mice. *$P < 0.05$. Values are mean ± s.e.m. n = 9-13.

5.4 DISCUSSION

5.4.1 *Effect of placement in a novel environment on behaviour in NK1+/+ and NK1-/- mice*

5.4.1.1 Effect of NK1 receptor disruption on behavioural response to novelty

Before ANCOVA analysis, these present studies found an effect of genotype on 5 behaviours. These included decreased ***risk assessment behaviours*** and ***time spent in the light zone*** in NK1-/- mice, whereas ***total spontaneous locomotor*** and ***rearing behaviours*** were increased in NK1-/- mice. A previous study, using the same strain of mice in the LDEB, found that after ANCOVA analysis, all of the 12 behaviours scored differed between genotype (Herpfer *et al.*, 2005).

Time spent in the light zone, latency to leave the light zone and the ***number of returns*** to the light zone are behaviours typically believed to represent active avoidance of an aversive stimulus. NK1-/- mice spend less time in the light zone, which may be due to an increase in active avoidance behaviour. Alternatively, disruption of the NK1 receptor could result in an increase in the time spent actively exploring the two compartments, with the overall effect that they spend less time in the light zone and have a higher locomotor activity. Since NK1+/+ mice spend more time performing risk assessment behaviours than NK1-/- mice, it is also possible that the higher amount of time spent in the light zone by NK1+/+ mice, is due their engagement in risk assessment behaviour.

In these studies, as shown previously (Herpfer *et al.*, 2005), NK1-/- mice exhibit higher spontaneous locomotor activity. Increased activity of the LC, and central noradrenergic neurotransmission, is believed to be critical for behavioural arousal (Carli *et al.*, 1983; Cole & Robbins, 1992; Robbins, 1997; Sauvage & Steckler, 2001). As shown in Chapter 3, halothane-anaesthetised NK1-/- mice possess higher basal NA efflux, demonstrating a difference in the regulation of the noradrenergic system between these two genotypes. A difference in the regulation of the noradrenergic system between NK1+/+ and NK1-/- mice may result in an increased sate of arousal, leading to increased spontaneous locomotor activity observed in NK1-/- mice. The higher

spontaneous locomotor activity in NK1-/- mice, could also contribute to the decreased time these mice spend in the light zone.

Rearing is generally interpreted as an exploratory behaviour. NK1-/- mice demonstrate an increase in this particular behaviour, compared to NK1+/+ mice. Stimulation of LC neurones electrically increases the time rats spend investigating / exploring novel objects (Devauges & Sara, 1991). Inhibition of the LC using an α_2-adrenoceptor agonist e.g. clonidine decreases this exploration because of sedation (Sara *et al.*, 1995), whereas, the α_2-adrencoceptor antagonist idazoxan enhances the response of LC neurones to novelty (Sara *et al.*, 1994). The increased rearing behaviour of NK1-/- mice could, therefore, be due to an increase in NA efflux, as shown in Chapter 3. After ANCOVA analysis, with locomotor activity as the covariate, the genotype dependent difference in rearing behaviour was abolished. The increase in rearing behaviour, therefore, is explained by an overall increase in the locomotor activity of the NK1-/- mice, which again could be attributed to the higher activity of the noradrenergic system in these mice.

The most striking effect of disruption of the NK1 receptor was seen on the risk assessment behaviours with NK1-/- mice performing fewer ***stretched-attend postures*** and spending less time in the ***flat back approach***. Stretched-attend postures and flat back approach reflect risk assessment in potentially threatening environments (Holmes *et al.*, 2001). The decreased risk assessment behaviours, in NK1-/- mice, suggest a decreased stress response to a novel environment. One possible explanation may be that disruption of the NK1 receptor modifies the noradrenergic system in such a way that NK1-/- mice are better able to cope with the novel adverse environment presented by the LDEB. Since noradrenergic transmission in the brain is essential for coping with stress (Anisman, 1985), it is possible that disruption of the NK1 receptor modifies the noradrenergic system so that NK1-/- mice are better able to 'cope' with the stressor novelty.

Alternatively it is possible that genetic disruption of the NK1 receptor impacts on other neurotransmitter systems, for example 5-HT, also. In NK1-/- mice, the 5-HT_{1A} receptor is down-regulated and desensitised (Froger *et al.*, 2001; Santarelli *et al.*, 2001). Risk assessment behaviours are decreased by 5-HT_{1A} antagonists (Rodgers & Cole, 1994).

The difference in risk-assessment behaviour may, therefore, be due to down-regulation of the 5-HT_{1A} receptor in NK1-/- mice

5.4.1.2 Effect of α_2-adrenoceptor antagonism independent of genotype

A number of behaviours were modified by RX821002 independent of genotype. These were an increase in the ***total number of grooms*** and ***total duration spent grooming***. Grooming is a complex and essential ritual to rodent behaviour (Kalueff & Tuohimaa, 2005). In rodents, grooming can represent a common attitude of body care, a displacement behaviour due to a stressful environmental situation and / or a behavioural pattern, expression of which is affected by specific damage to its neural substrates (for review see: (Spruijt *et al.*, 1992)). Mouse grooming can be altered by various stressors, and is thought to be a coping strategy to deal with the stressful situation (Dantzer, 1993), which could explain the increase in this particular behaviour.

Time spent in ***flat back approach*** and the number of ***stretched-attend postures*** in RX821002 pre-treated NK1-/- mice were slightly increased and decreased respectively. In NK1+/+ mice RX821002 decreased both flat back approach and stretched-attend postures. Since RX821002 binds to 5-HT_{1A} receptors where it acts as an antagonist (K_i 20-30 nM), (Grijalba *et al.*, 1996; Newman-Tancredi *et al.*, 1998; Ogilvie & Clarke, 1998), and previous studies have shown that 5-HT_{1A} receptor antagonists decrease risk assessment behaviours (Rodgers & Cole, 1994), it is possible that binding of RX821002 to 5-HT_{1A} is a confounding factor in these studies.

These effects of RX821002 independent of genotype, do suggest that RX821002 is having some residual effect on α_2-adrenoceptors in both NK1+/+ and NK1-/- mice. It is possible that these effects are mediated through α_2-adrenoceptor expressed on neurones which do not express NK1 receptors, since it is possible that α_2-adrenoceptors are modified only on those neurones which co-express α_2-adrenoceptors and NK1 receptors.

5.4.1.3 Effect of α_2-adrenoceptor antagonism dependent on genotype

It is a generally held view that an increase in NA release in the brain is one of the underlying causes of anxiety (Redmond, Jr. & Huang, 1979). There is also evidence to

challenge this idea, that suggests that an increase in LC activity that accompanies stressful situations may also acts as a compensatory, coping mechanism (Weiss *et al.*, 1994).

Before ANCOVA analysis, significant 'drug x genotype' interactions were observed on ***time spent grooming in the light*** and ***time to return to the light zone***. After taking into consideration individual differences in locomotor activity, an effect of RX821002 in NK1+/+ mice, only, was also observed on ***number of returns to the light zone***, ***number of rears in the dark*** and ***latency to leave the light zone***. This suggests that the higher locomotor behaviour, in NK1-/- mice, was masking effects of RX821002 dependent on genotype.

Although the LDEB, in these studies, was not used to determine anxiogenic / anxiolytic responses it is interesting to note that anxiolytics e.g. BDZs increase exploratory rearing behaviour of rodents in the dark zone (Costall *et al.*, 1989; Shimada *et al.*, 1995). The marked decrease in rearing behaviour in the dark zone in RX821002 pre-treated NK1+/+ mice, but not in NK1-/- mice, may, therefore, suggest an anxiogenic effect of this α_2-adrenoceptor antagonist observed only in NK1+/+ mice. Anxiolytic BDZs, also increase the latency to leave the light zone of mice in the LDEB (Costall *et al.*, 1989). Here, NK1+/+ mice treated with RX821002 demonstrate a marked increase in the latency to leave the light zone. In contrast, this behaviour was unaffected by RX821002 in NK1-/- mice. There are two possible explanations for these findings. There could be a decrease in anxiety-like behaviour so that NK1+/+ pre-treated mice spend more time exploring the novel compartment. This could account for the increase in latency to leave the light zone. Alternatively it is likely that the novel environment increases the activity of the LC-noradrenergic system. This increase in noradrenergic activity would be potentiated by systemic administration of the α_2-adrenoceptor antagonist RX821002. An increase in central noradrenergic activity induced by RX821002, could lead to feelings of alarm or fear (Redmond, Jr. & Huang, 1979) or vice versa. Fear can elicit a freezing behaviour in rodents (Graeff, 1994). Ethologically, freezing behaviour can be thought of as one type of predator avoidance reaction (Blanchard *et al.*, 1975; Takahashi *et al.*, 2005). The level of fear could dictate the animals response, and it is possible that an increase in novelty may turn exploratory behaviours or escape reactions into freezing (see: (Haapalinna *et al.*, 1999)), leading to the increased latency to leave the light zone

in the NK1+/+ mice, supporting an anxiogenic like effect of RX821002 in NK1+/+ mice only.

The use of α_2-adrenoceptor antagonists, in these current studies, enabled identification of certain genotype-dependent behavioural differences that could be attributed to a difference in the regulation of the noradrenergic nervous system. ***Time to return to the light zone*** after first exit, is generally viewed as passive avoidance behaviour. α_2-Adrenoceptor antagonists e.g. RX821002 and atipamezole decrease the time to return to the light zone in NK1+/+ mice, so that they are indistinguishable from NK1-/- mice pre-treated with either saline or the α_2-adrenoceptor antagonist (Fisher *et al.*, 2003; Fisher *et al.*, 2004). Yohimbine decreases time to return to the light zone after first exit in NK1+/+ mice, only, so that they are indistinguishable from NK1-/- mice treated with vehicle (Stewart *et al.*, 2002). Therefore, of the 12 behaviours scored during the LDEB, ***time to return to the light zone*** is the one of the most likely behaviours that can be attributed to a difference in the function of the noradrenergic system between NK1+/+ and NK1-/- mice.

Grooming in rodents is increased by arousing / stressful conditions e.g. novelty (See: (Spruijt *et al.*, 1992). The marked increase in time spent grooming in the novel light compartment was observed in NK1+/+ but not NK1-/- mice, following pre-treatment with RX821002. It is possible that systemic administration of RX821002 potentiated the stress induced release of NA in response to novelty in NK1+/+ mice, only. Via activation of α_1-adrenoceptors, this may facilitate ACTH secretion and an increase in grooming behaviour (Cecchi *et al.*, 2002b; Cecchi *et al.*, 2002a).

5.4.2 Microdialysis measurement of noradrenaline efflux in a novel environment

The studies describe, for the first time, the effect of placement in a novel compartment on NA efflux in mice. Experiments conducted previously in this laboratory, on rats, have shown that placement in a novel environment increases NA efflux (Dalley & Stanford, 1995; Dalley *et al.*, 1996; McQuade *et al.*, 1999), as does a conditioned cue for placement in the aversive environment (McQuade & Stanford, 2000).

5.4.2.1 *Effect of NK1 receptor disruption on noradrenaline efflux in the light / dark exploration box*

No genotype dependent difference in NA efflux was found when animals were placed either in the dark zone or the novel light zone. What is interesting to note, however, is the difference in basal NA efflux between mice placed in the dark zone to habituate, and those animals that had been left in their home cage over night and in which the experiment was conducted (Chapter 4). The average basal efflux of NK1+/+ mice left in their home cage was 4.72 ± 0.34 fmol / 20 min, compared with 9.88 ± 2.06 fmol / 20 min in animals placed in the dark zone of the LDEB to habituate. The average basal NA efflux in NK1-/- mice left in their home cage was 5.22 ± 0.36 fmol / 60 min, compared with 6.77 ± 1.23. However, as these two experiments were not fully randomised with each other it is not possible to draw direct comparisons between the two.

5.4.2.2 *Effect of saline and RX821002 pre-treatment on noradrenaline efflux in the light / dark exploration box*

Analysis of the raw microdialysis data revealed that pre-treatment with RX812002 increased NA efflux in NK1+/+ mice, only. This increase in NA efflux occurred before the mice were transferred to the novel light compartment. Pre-treatment with saline did not increase NA efflux in either genotype. The effect of the saline injection on NA efflux is rather variable, with some studies reporting an increase (Dalley *et al.*, 1996) or no effect on NA efflux following saline injection (Mason *et al.*, 1998).

However, analysis of net data showed no difference in the net increase in NA efflux following either RX821002 or saline pre-treatment. Suggesting that the higher basal NA efflux in the NK1+/+ mice destined for RX821002 treatment was responsible for the observed increase in NA efflux when the raw data was analysed.

5.4.2.3 *Effect of placement in the light novel compartment on noradrenaline efflux*

The only group which demonstrated an increase in NA efflux following placement in the light novel compartment were NK1+/+ mice pre-treated with RX821002. However,

the net increase in RX821002 pre-treated mice was not different between groups, again suggesting that the increase in NA efflux observed with the raw data was due to a higher basal NA efflux in this group of mice. The increase in NA efflux observed in NK1+/+ mice treated with systemic RX821002 dissipated after the first 60 min of placement in the novel arena. This suggests an habituation to the novel compartment which is in agreement with previous microdialysis studies (Dalley *et al.*, 1996; Dalley & Stanford, 1995; McQuade & Stanford, 2000; McQuade *et al.*, 1999) and studies demonstrating that the increase in activity of the LC-noradrenergic system declines following repeated exposure to a sensory stimulus as the animals interest in them, or attention to them, declines (Jacobs, 1986; Rasmussen *et al.*, 1986; Vankov *et al.*, 1995; Herve-Minvielle & Sara, 1995). Furthermore, the increase in NA efflux in RX821002 pre-treated NK1+/+ mice over the first 60 min of placement in the LDEB corresponds with the period during which behavioural monitoring took place, suggesting that the increase in NA efflux could contribute to behavioural changes induced by RX821002 in NK1+/+ mice: e.g. decreased time to return to the light zone and increased time to leave the light zone.

5.4.3 Caveats

The lack of 'genotype x drug' interaction of RX821002 on net NA efflux but an obvious effect on the raw NA concentration in the frontal cortex of NK1+/+ mice suggests the variance of the sample masked the anticipated change, and that the n number should be increased. However, performing the Mead's (1988) 'Resource equation' on this experiment proves sufficient numbers of animals were used (see: Festing *et al.*, 2002). Therefore, it may be possible to say that there is a biological change but it just misses statistical significance.

The behavioural experiments and the *in vivo* microdialysis experiments carried out in the LDEB were performed separately. Previous studies conducted in this laboratory have demonstrated that implantation of a probe modifies the behavioural response to certain environmental stimuli (McQuade *et al.*, 1999). These studies suggest probe implantation intensifies the behavioural response to a novel environment. Therefore, future studies could investigate the behavioural and NA efflux response of NK1+/+ and NK1-/- mice to the LDEB in a fully randomised experiment.

5.4.4 Summary and conclusions

The behavioural response to novelty, a non-noxious naturalistic environmental stressor, is affected by genetic disruption of the NK1 receptor. NK1-/- mice demonstrate an increase in spontaneous exploratory behaviours e.g. rearing behaviour and locomotor behaviour. A decrease in active avoidance behaviour e.g. time spent in the light zone and a decrease in risk assessment behaviours e.g. stretched-attend postures and time in flat back approach is also found with NK1-/- mice.

Systemic RX821002 produces a number of behavioural changes independent of genotype:

Total number of grooms
Total time spent grooming
Time spent in the light zone
} ↑ In both NK1+/+ and NK1-/- mice.

Number of stretched-attend postures
Time spent in flat back approach
} ↓ In both NK1+/+ and NK1-/- mice, although time spent in flat back approach appeared slightly increased or unchanged in NK1-/- mice.

Systemic RX821002 produces a number of behavioural changes dependent of genotype:

Time to return to the light zone
Rears in the dark zone
Returns to the light zone
} ↓

Time spent grooming
Latency to leave the light zone
} ↑ These behavioural changes are observed in the NK1+/+ mice only, supporting the hypothesis that in NK1-/- mice the α_2-adrenoceptor is impaired in some way.

Placement of NK1-/- mice in a novel compartment does not result in an increase in NA efflux in either saline or RX821002 pre-treated animals. In contrast, placement of NK1+/+ mice in a novel environment increases NA efflux but only following systemic

administration of RX821002. However, further experiments using microdialysis in the LDEB are required to confirm that this increase in NA efflux is not purely the result of an increase in basal NA efflux in this group of mice.

The next chapter describes experiments investigating the localisation and density of the enzymes involved in the synthesis of NA, along with the noradrenaline transporter (NAT), involved in the reuptake and inactivation of NA. A difference in the density of these enzymes or the NAT could contribute to the difference in basal NA efflux in NK1+/+ and NK1-/- mice. Studies in the following chapter also investigate the density and functional activity of the α_2-adrenoceptor, the prime candidate underlying the difference in NA efflux between NK1+/+ and NK1-/- mice.

CHAPTER 6

INVESTIGATING α_2-ADRENOCEPTOR DISTRIBUTION AND FUNCTION IN NK1+/+ AND NK1-/- MICE

6 INVESTIGATING α_2-ADRENOCEPTOR DISTRIBUTION AND FUNCTION IN NK1-/- AND NK1+/+ MICE

6.1 INTRODUCTION

This chapter presents results from a series of experiments profiling the localisation and density of α_2-adrenoceptors, using a combination of molecular approaches including immunohistochemistry (IHC), Western blot protein analysis and [^{3}H]RX821002 receptor autoradiography. The functional activity of α_2-adrenoceptors was investigated using adrenaline-stimulated [^{35}S]GTPγS binding. Noradrenergic neuronal markers: e.g. dopamine-β-hydroxylase (DβH), tyrosine hydroxylase (TH) and the noradrenaline transporter (NAT) are also compared in NK1+/+ and NK1-/- mice.

6.1.1 Background

Although the noradrenergic system of the rat has been extensively mapped (Svensson, 1982; Maeda *et al.*, 1991; Iijima, 1993), little is known about its distribution in the mouse CNS. Therefore, one of the aims of this project was to identify noradrenergic neurones in both the frontal cortex (M2) region, at the level of probe implantation, and the locus coeruleus (LC), using noradrenergic neuronal markers in NK1-/- and NK1+/+ mice. Methods chosen included immunohistochemical localisation of the enzymes DβH and TH. The distribution of NA terminals in the cortex is most clearly demonstrated by the immunocytochemical localisation of DβH, the enzyme responsible for conversion of dopamine (DA) to noradrenaline (NA) in the biosynthetic pathway. TH, the rate-limiting enzyme in the synthesis of adrenaline, NA and DA, is also a valuable marker for mapping the noradrenergic system. However, it is found in adrenergic, noradrenergic and dopaminergic neurones, so DβH is the more specific marker for the noradrenergic system. It is important to note that DβH is also found in adrenaline containing neurones, but, there are only a few present in the rodent brain, projecting in low density to forebrain regions.

As described in Section 1.4.3 the NAT is responsible for the clearance of NA from the synaptic cleft. The NAT is found on noradrenergic neurones, only (Lorang *et al.*, 1994).

This makes it a suitable marker with which to map noradrenergic neurones in NK1+/+ and NK1-/- mice.

Previous microdialysis studies showed a 4-5–fold increase in NA efflux in the frontal cortex of NK1-/- compared with their wild-type counterparts (Chapter 3). The hypothesis was that this may be due to a downregulation or desensitisation of autoregulatory α_2-adrenoceptors either in the frontal cortex (FCtx) or LC. The M2 region of the FCtx was chosen for analysis of the α_2-adrenoceptors (function and density) as the microdialysis probe was placed in this brain area. As described in Section 1.4.4, agonist activation of α_2-adrenoceptors decreases noradrenergic neuronal firing-rate and release. A decrease in either the density or functional activity of these receptors could be the cause of, or contribute to, the increase in NA efflux observed in NK1-/- mice. This would also echo the findings of Froger and Santarelli (Froger *et al.*, 2001; Santarelli *et al.*, 2001), who demonstrated a decrease in both the density and function of autoregulatory 5-HT_{1A} receptors in NK1-/- mice. Downregulation and desensitisation of 5-HT_{1A} autoreceptors, results in an increase in the firing-rate of dorsal raphé (DR) neurones and an increase in 5-HT release in NK1-/- mice following treatment.

It is also possible that there is a difference in the amount / activity of the enzymes involved in the synthesis of NA (DβH and TH). The use of these enzymes as markers for mapping the noradrenergic system of the M2 region of the frontal cortex and LC in NK1-/- and NK1+/+ mice also enabled analysis of whether there was an observable difference in the density of these two enzymes, which could account for the difference in NA efflux. It is, however, important to note that a difference (or lack of) in the density of the enzymes involved in the synthesis of NA does not provide an index for their activity.

A number of approaches were taken to investigate α_2-adrenoceptor protein density and function. These included: preliminary IHC analysis; Western blot protein analysis; radioligand receptor autoradiography; and measurement of adrenaline-stimulated [^{35}S]GTPγS binding to brain sections of NK1+/+ and NK1-/- mice to investigate the coupling of α_2-adrenoceptors to G proteins. This approach was based on a study by

Froger *et al.,* (2001) who compared the autoregulatory pre-synaptic 5-HT_{1A} receptor, in the same background strain of NK1-/- and NK1+/+ mice (Froger *et al.*, 2001).

By analogy with the results from Froger *et al.,* (2001), it was predicted that the increase in basal NA efflux, in anaesthetised NK1-/- mice, could be attributed to either a downregulation and / or desensitisation of α_2-adrenoceptors. This could be occurring at either the noradrenergic terminals in the frontal cortex, where they act to decrease release of NA, somatodendritically, at the level of the LC, where they act to decrease firing-rate, or both. A decrease in the binding density of [^{3}H]RX821002 and / or a decrease in [^{35}S]GTPγS adrenaline-stimulated binding in NK1-/- mice would support this proposal.

6.2 PROTOCOLS

6.2.1 Mice

All experiments were conducted on tissue from adult male NK1-/- and NK1+/+ mice (n = 3-8 per genotype).

6.2.2 Immunohistochemistry

As described in Section 2.8 and Appendix 1.

6.2.3 Western blot protein analysis

As described in Section 2.9.

6.2.4 [^{3}H]RX821002 and [^{3}H]rauwolscine receptor autoradiography

As described in Section 2.10.

6.2.5 Adrenaline-stimulated [^{35}S]GTPγS binding assay

As described in Section 2.11.

6.2.6 Data analysis

Data obtained from Western blot protein analysis, [^{3}H]RX821002 ligand binding and [^{35}S]GTPγS were analysed using an independent samples *t*-test. [^{35}S]GTPγS data were also analysed using the Wilcoxon matched-pairs signed-ranks test.

6.3 RESULTS

6.3.1 Immunohistochemical localisation of the α_{2a}-adrenoceptor, dopamineβ-hydroxylase, noradrenaline transporter and tyrosine hydroxylase in the frontal cortex and locus coeruleus of NK1+/+ and NK1-/- mice

The initial IHC for DβH, the NAT and TH revealed extensive localisation of the enzymes and transporter in both the M2 region of the FCtx, (Figure 6.1a-f), and LC (Figure 6.2a-d). However, no differences between the genotypes were found. DβH and TH were used as histochemical markers to map the location of the LC (Figure 6.2a-d). IHC studies confirmed the expression of NK1 receptors on LC neurones (Figure 6.3). The initial immunohistochemical findings suggest that there are fewer α_{2a}-adrenoceptors in the LC of NK1-/- mice compared with their wildtype counterparts (Figure 6.4a-b).

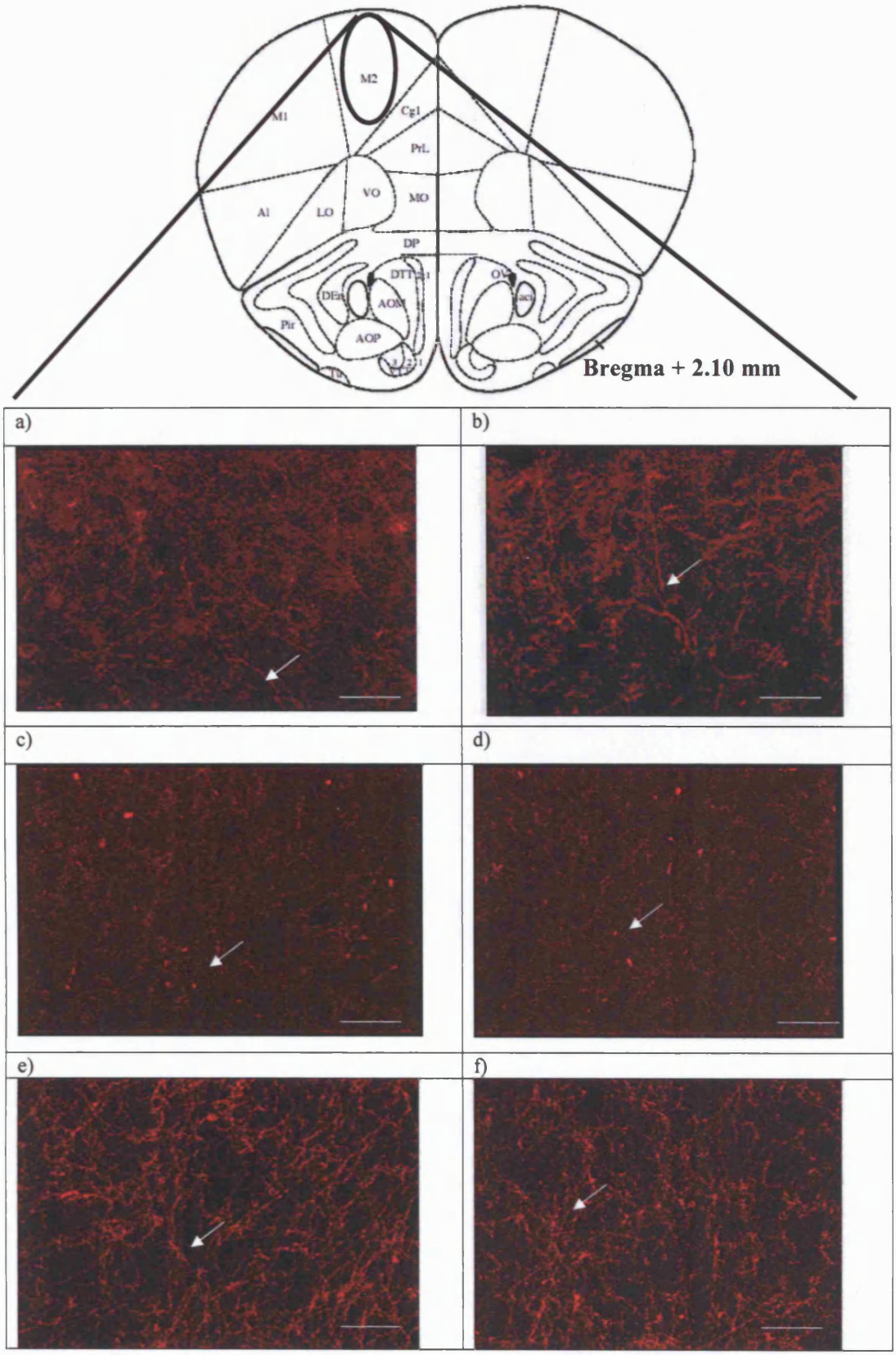


FIGURE 6.1a-f: Dopamine-β-hydroxylase in a) NK1+/+ and b) NK1-/- mice. Noradrenaline transporter in c) NK1+/+ and d) NK1-/- mice. Tyrosine hydroxylase in e) NK1+/+ and f) NK1-/- mice. X 40 magnification at the level of the frontal cortex (M2 region), scale = 10μM. Arrows indicate fibers.

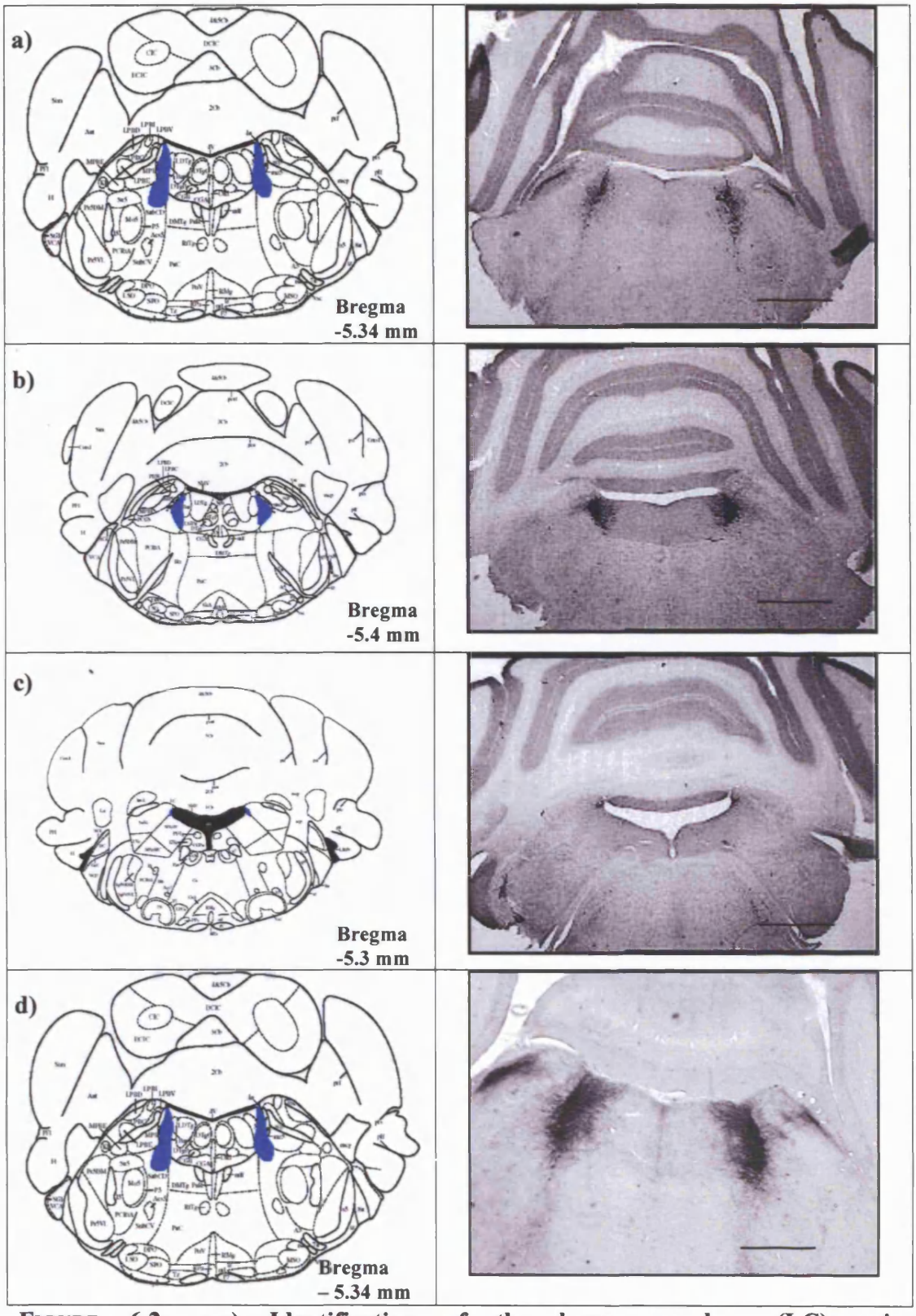


FIGURE 6.2: a-c) Identification of the locus coeruleus (LC) using dopamine-β-hydroxylase, X 1.6 magnification, scale = 1 mm, and d) tyrosine hydroxylase X 5 magnification, scale = 1 mm, as the histochemical marker with DAB as the visualisation reaction. The LC is labelled blue in the mouse brain atlas images.

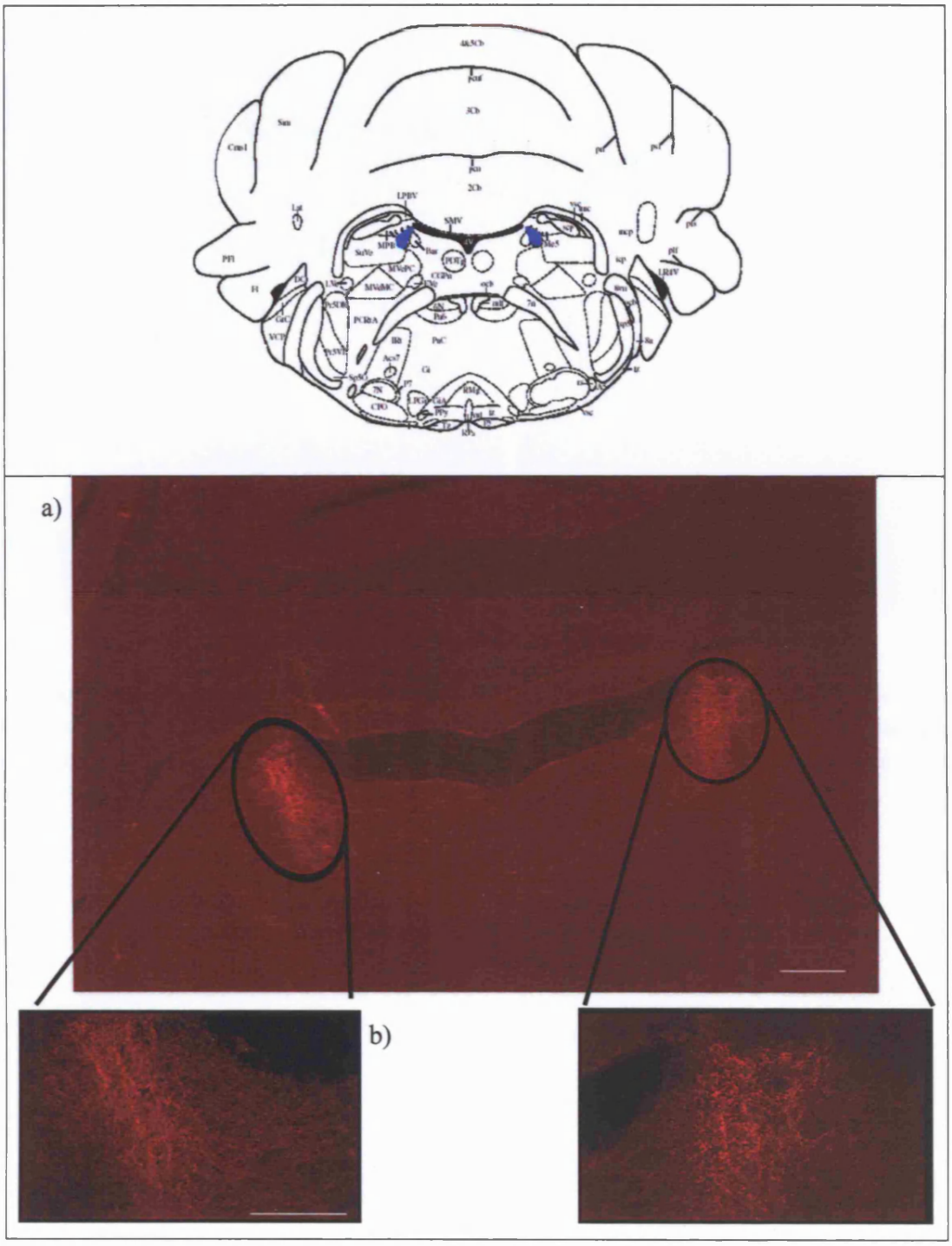


FIGURE 6.3: Expression of NK1 receptors in the locus coeruleus a) X 10 magnification, scale = 3 mm and b) X 20 magnification, scale = 0.25 mm, with Cy3 as the visualisation reaction.

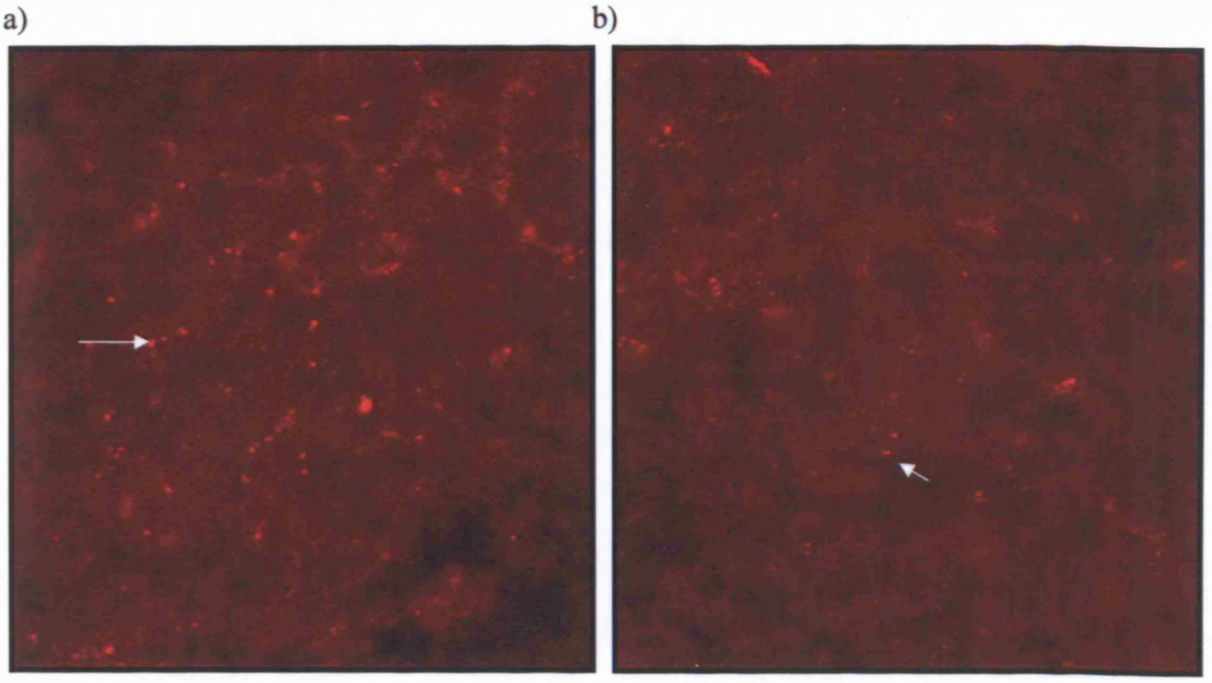


FIGURE 6.4: Expression of α_{2a}-adrenoceptors in the locus coeruleus (LC) of a) NK1+/+ and b) NK1-/- mice. Preliminary immunohistochemistry of the LC suggested a reduced density of α_{2a}-adrenoceptors in the LC of NK1-/- mice X 40 magnification. Arrows indicate α_{2a}-adrenoceptor labelling.

6.3.2 Western blot analysis of α_{2a}-adrenoceptor and noradrenaline transporter protein

There was no difference in either α_{2a}-adrenoceptor or NAT protein in the FCtx, Hipp or LC of NK1+/+ and NK1-/- mice (Figure 6.5a-c; Table 6.1).

Amount of α_{2a}-adrenoceptor and NAT protein expressed as percentage of NK1+/+ mice			
α_{2a}-adrenoceptor	**FCtx %**	**Hipp %**	**LC %**
NK1+/+	100 ± 1.79	100 ± 1.54	100 ± 3.34
NK1-/-	99.87 ± 2.25	101.37 ± 1.95	107.31 ± 2.67
NAT	**FCtx %**	**Hipp %**	**LC %**
NK1+/+	100 ± 22.75	100 ± 12.32	100 ± 8.94
NK1-/-	83.64 ± 11.49	132 .69 ± 25.69	117.23 ± 5.88

TABLE 6.1: Western blot protein analysis of the amount of α_{2a}-adrenoceptor and NAT protein in the FCtx, Hipp and LC of NK1+/+ and NK1-/- mice. Values are mean ± s.e.m. (n = 5-6), expressed as a percentage of the density of protein found in NK1+/+ mice. FCtx: frontal cortex; Hipp: hippocampus; LC: locus coeruleus; NAT: noradrenaline transporter.

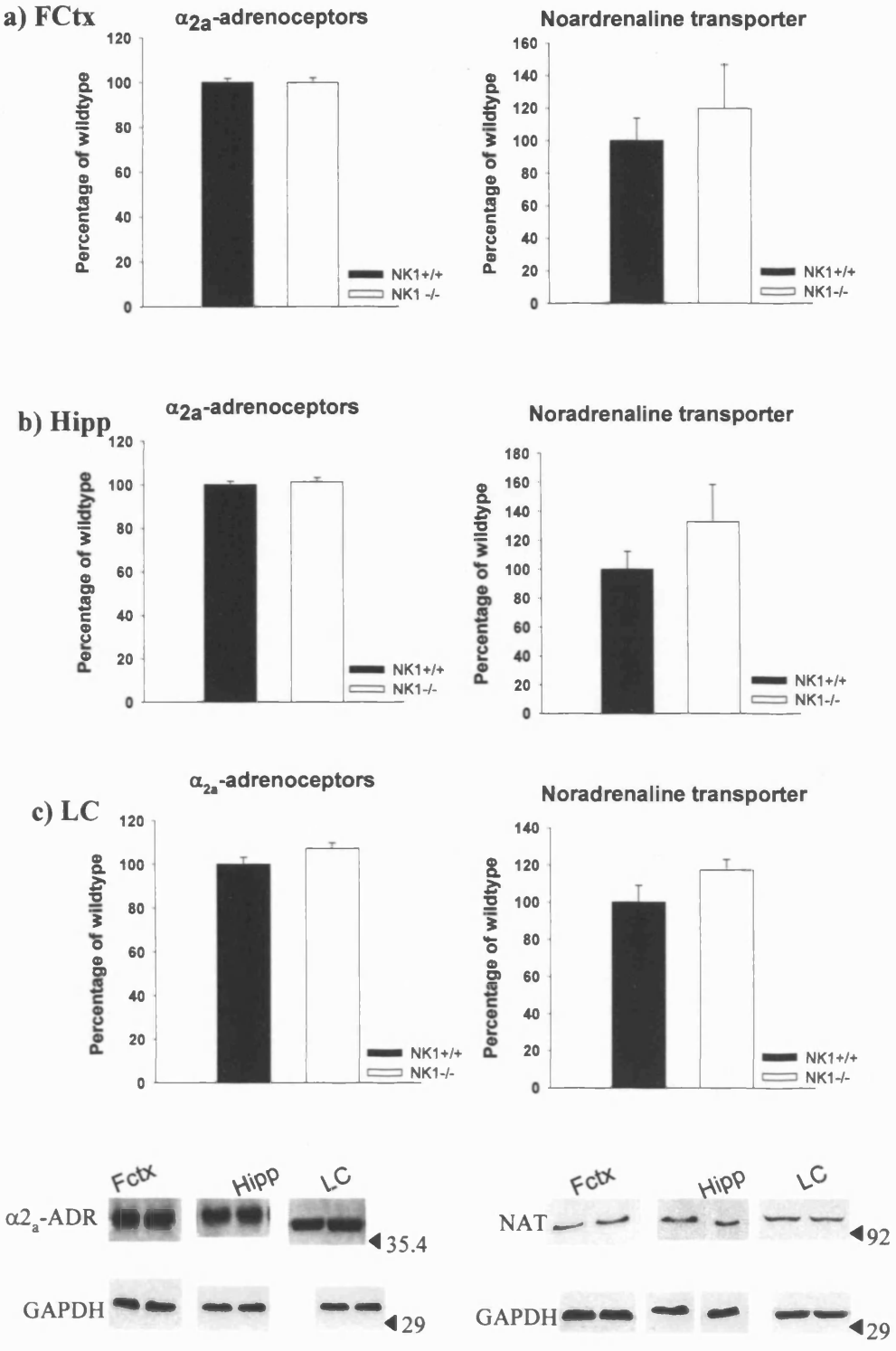


FIGURE 6.5a-c: Western blot protein analysis of α_{2a}-adrenoceptors and the noradrenaline transporter (NAT) in the frontal cortex (FCtx), hippocampus (Hipp) and locus coeruleus (LC). Values are presented as percentage of NK1+/+ mice from n = 5-6.

6.3.3 Quantitative α_2-adrenoceptor autoradiography with [^{3}H]RX821002 and [^{3}H]rauwolscine in NK1+/+ and NK1-/- mice

In these studies, receptor density was assessed at an approximate K_d (single concentration point) for the radioligand.

[^{3}H]RX821002 binding was found throughout the brain (Figure 6.6a-d). Limbic brain regions, receiving a dense noradrenergic innervation from the LC, were also analysed. These included: the amygdala (amyg), bed nucleus stria terminalis (BNST), cerebellum (CBM), hippocampus (Hipp), lateral septum (LSI), locus coeruleus (LC), olfactory bulbs (OB), periaqueductal grey (PAG) and the frontal cortex (M2 region). Areas with the highest density of α_{2a}-adrenoceptors were: the LSI, BNST and PAG (Table 6.2). The binding density in all these brain regions was not different in NK1+/+ and NK1-/- mice. In particular, no difference was found between NK1+/+ and NK1-/- in the area of the LC and FCtx (Figure 6.7a-b). The lowest binding density of [^{3}H]RX821002 in both genotypes was found in the CBM (Table 6.2). Binding of the α_{2c}-adrenoceptor-preferring radioligand, [^{3}H]rauwolscine, was above film background only in the caudate putamen, area CA1 of the hippocampus and the nucleus accumbens (Figure 6.6e-f).

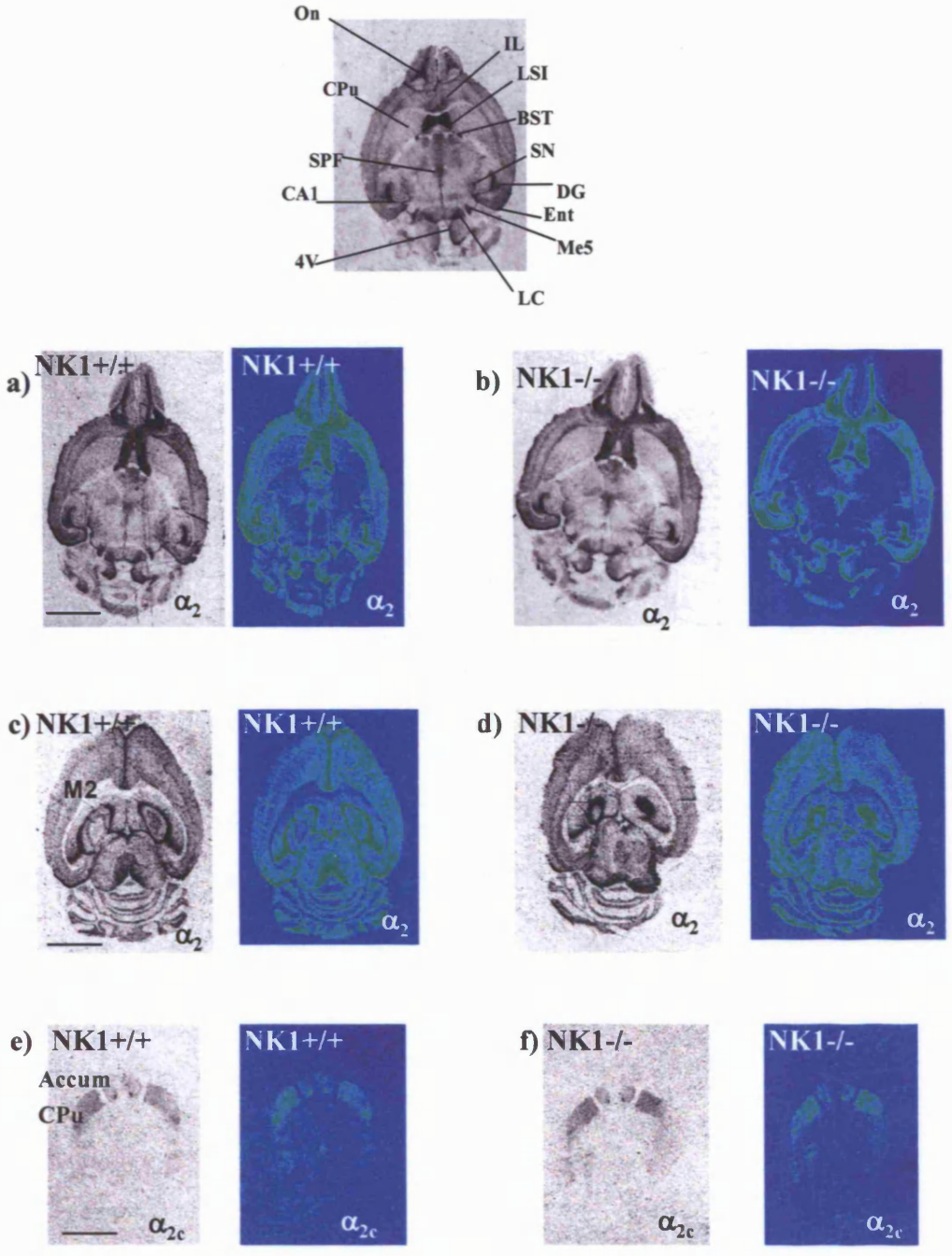


FIGURE 6.6: a-d) [^{3}H]RX821002 and e-f) [^{3}H]rauwolscine binding in horizontal sections of NK1+/+ and NK1-/- mice. The selected autoradiographic images (a-d) show the distribution of α_2-adrenoceptor [^{3}H]RX821002 binding in the olfactory nucleus (On), lateral septum (LSI), striatum (CPu), bed nucleus of the stria terminalis (BNST), subparafasicular thalamic nuclei (SPF), caudal CA1 region of the hippocampus (CA1), entorhinal cortex (Ent), mesencephalic trigeminal nucleus (Me5) and locus ceruleus (LC), Frontal cortex (FCtx), M2 region. Binding of the α_{2a}-adrenoceptor preferring radioligand, [^{3}H]rauwolscine, was found only in the striatum (CPu) and Accumbens (nucleus accumbens) and CA1 region of the hippocampus. Receptor density is expressed as fmol / mg wet tissue. Values are mean ± s.e.m. For each region, bilateral measurements were made from at least three brain sections. N = 3. Scale bar = 3.5 mm.

Brain region	Receptor density (fmol / mg tissue ± s.e.m.)	
	NK1+/+	NK1-/-
Amygdala	50.02 ± 22.9	53.42 ± 26.6
Bed nucleus stria terminalis	107.39 ± 18.47	83.06 ± 12.72
Cerebellum (Granule cell layer and molecular layer)	18.98 ± 14.77	20.09 ± 15.31
Hippocampus (CA1, CA2, CA3, dentate gyrus, subiculum, parasubiculum and presubiculum)	58.15 ± 7.77	47.48 ± 13.51
Locus Coeruleus	100.47 ± 9.1	92 ± 10.48
Lateral Septum (dorsal, intermediate and ventral part)	94.39 ± 8.15	95.99 ± 6.22
Frontal cortex (M2)	63.49 ± 37.57	68.55 ± 33.72
Entire olfactory bulb	63.02 ± 17.59	53.35 ± 11.85
Periaqueductal Grey	92.48 ± 16.75	70.99 ± 3.12

TABLE 6.2: α_{2a}-Adrenoceptor density was measured in brain regions by quantitative autoradiography using 1 nM [^{3}H]RX821002 as ligand. Values represent specific binding. Receptor density is expressed as fmol / mg tissue ± s.e.m. and represents mean determinations of at least three sections from n=3 animals. Non-specific binding was determined in the presence of 100 μM RX821002. Non-specific binding was not detected on autoradiographs and so film background was taken as non-specific binding (approximately 1-2 fmol / mg tissue).

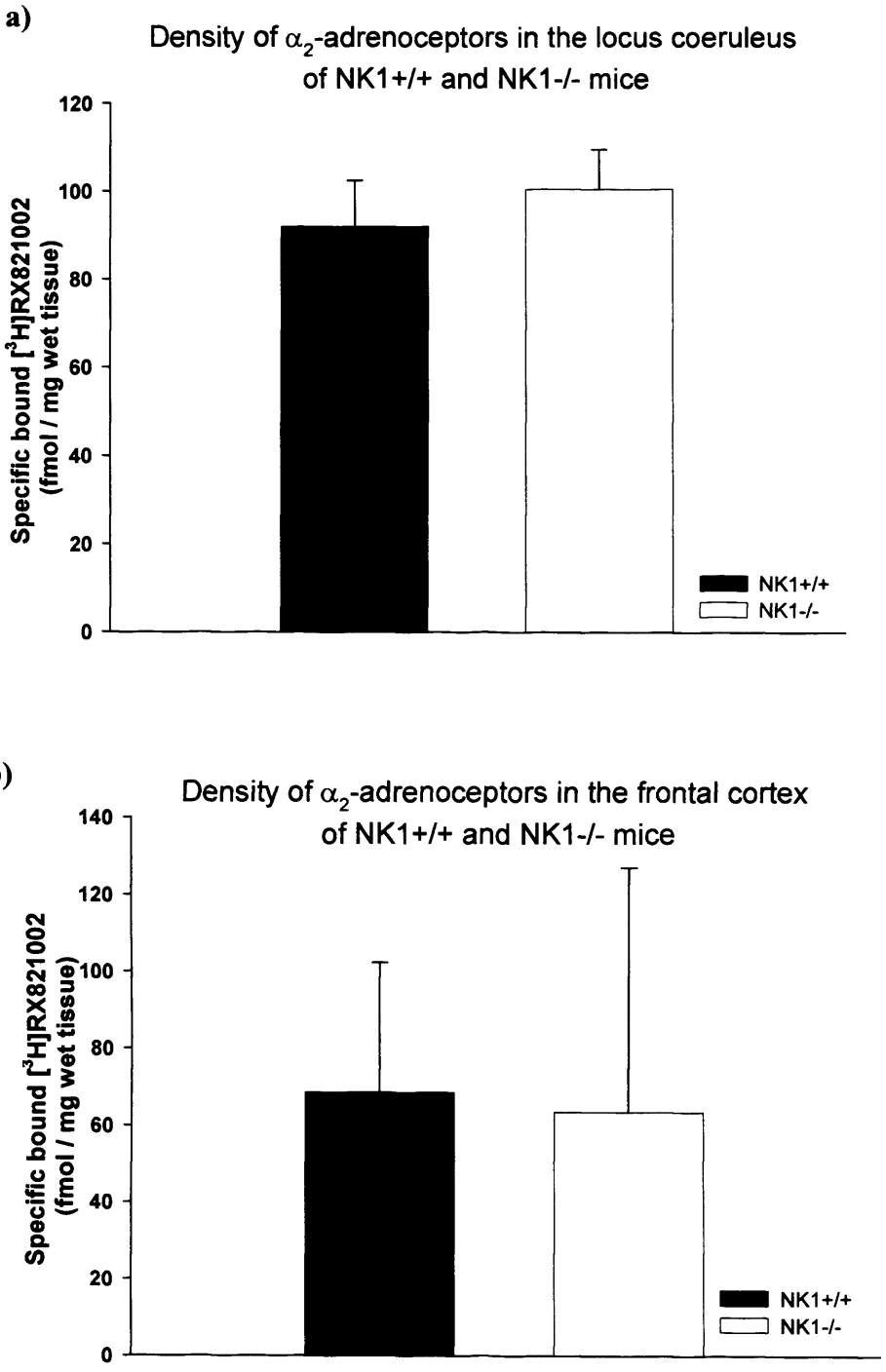


FIGURE 6.7: Bar chart depicting quantified differences between NK1+/+ and NK1-/- mice in a) the locus coeruleus and b) the frontal cortex (M2 region), n = 3.

6.3.4 Analysis of the functional activity of α_2-adrenoceptors in NK1+/+ and NK1-/- mice using adrenaline-stimulated [^{35}S]GTPγS binding

Optical density measurements of [^{35}S]GTPγS autoradiographic measurement were made within the LC and M2, under four experimental conditions:

- Non-specific
- Basal
- Adrenaline-stimulated
- RX821002 alone

In both NK1+/+ and NK1-/- mice, adrenaline (100 μM) increased [^{35}S]GTPγS labelling (Table 6.3, Figure 6.8). Adrenaline stimulated [^{35}S]GTPγS binding in most brain regions, except within white matter, mesencephalon and CBM (Figure 6.8c-d). Adrenaline-stimulated [^{35}S]GTPγS binding was reduced to basal levels by the specific α_2-adrenoceptor antagonist RX821002 (100 μM; Figure 6.8a-f). RX821002 alone, did not appear to have any observable effects on basal adrenoceptor activity (Figure 6.8a-f).

The pattern of adrenaline-stimulated [^{35}S]GTPγS binding corresponds well with the distribution of α_2-adrenoceptor binding sites, as determined by receptor autoradiography using [^{3}H]RX821002 (Figure 6.7a-d). High binding densities of α_2-adrenoceptor and adrenaline-stimulated [^{35}S]GTPγS binding are found in the LSI, BNST, DG, mesencephalic trigeminal nucleus, and LC (Figure 6.7a-b and Figure 6.8c-d). Moderate densities of both markers were found in the striatum and entorhinal cortex, substantia nigra, field CA1 of Ammon's horn and throughout the cerebral cortex. Low / negligible values of both markers are found in the CBM, white matter tracts and mesencephalon (Figure 6.7a-b and Figure 6.8c-d).

Comparison of the genotypes showed no difference in the adrenaline-stimulated [^{35}S]GTPγS binding in either the LC (Figure 6.8c-d; Table 6.3) or frontal cortex (M2 region) (Figure 6.8e-f; Table 6.3), the main areas of interest in these studies. However, it is interesting to note that NK1-/- mice demonstrated lower adrenaline-stimulated [^{35}S]GTPγS binding in 5 out of the 8 films analysed (Figure 6.9).

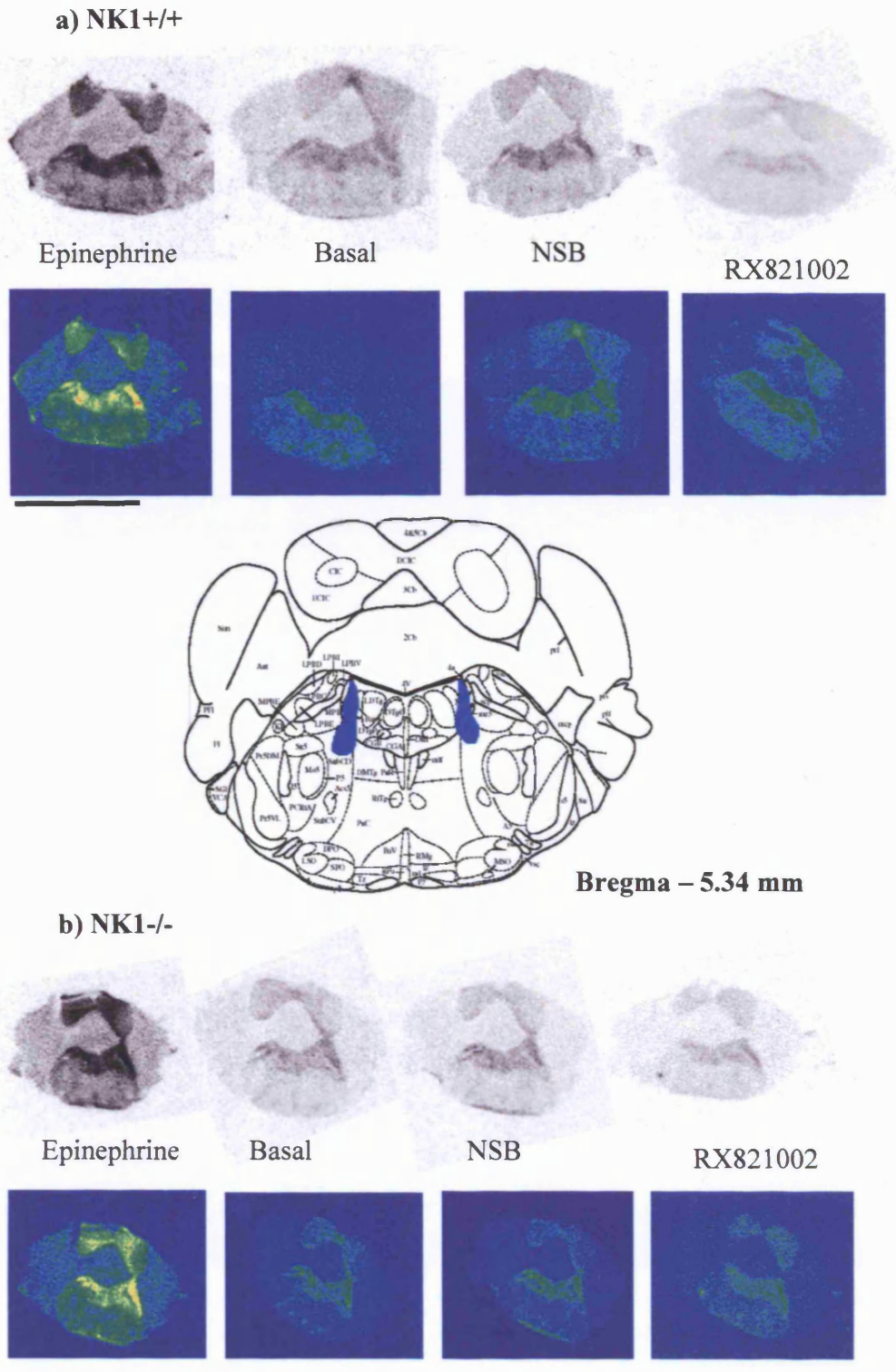


FIGURE 6.8a-b: Representative autoradiographs of α_2-adrenoceptor mediated increase in [^{35}S]GTPγS binding to brain sections from a) NK1+/+ and b) NK1-/- mice at the level of the locus coeruleus. Coronal (15μm) sections were labeled by [^{35}S]GTPγS (0.1 nM), without (basal) or with 100μM epinephrine, at the level of the LC. Nonspecific (NSB) labeling was obtained from adjacent sections exposed to 100 μM epinephrine plus 100 μM RX821002. n = 8. The area of the locus coeruleus can be identified as labelled blue in the Paxinos and Watson images. Scale = 4000 μm.

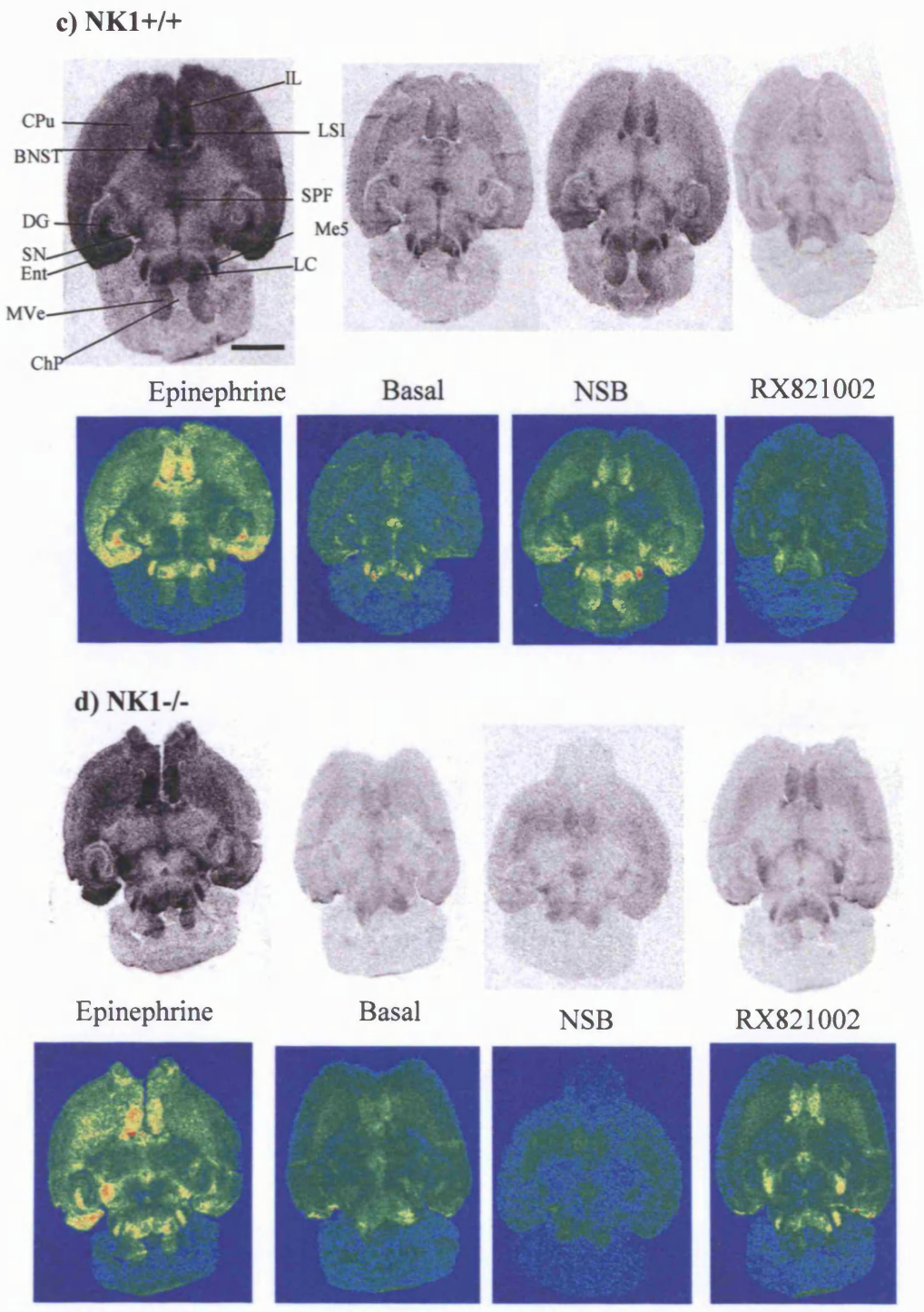


FIGURE 6.8c-d: Representative autoradiographs of α_2-adrenoceptor mediated increase in [^{35}S]GTPγS binding to horizontal brain sections from c) NK1+/+ and d) NK1-/- mice, at the level of the locus coeruleus. Infralimbic cortex (IL), lateral septum (LSI), subparafasicular thalamic nuclei (SPF), mesencephalic trigeminal nucleus (Me5), locus ceruleus (LC), caudate putamen (CPu), bed nucleus of the stria terminalis (BNST), dentate gyrus (DG), substantia nigra (SN), entorhinal cortex (ent), medial vestibular nucleus (MVe), choroid plexus (ChP). Scale = 3.5mm.

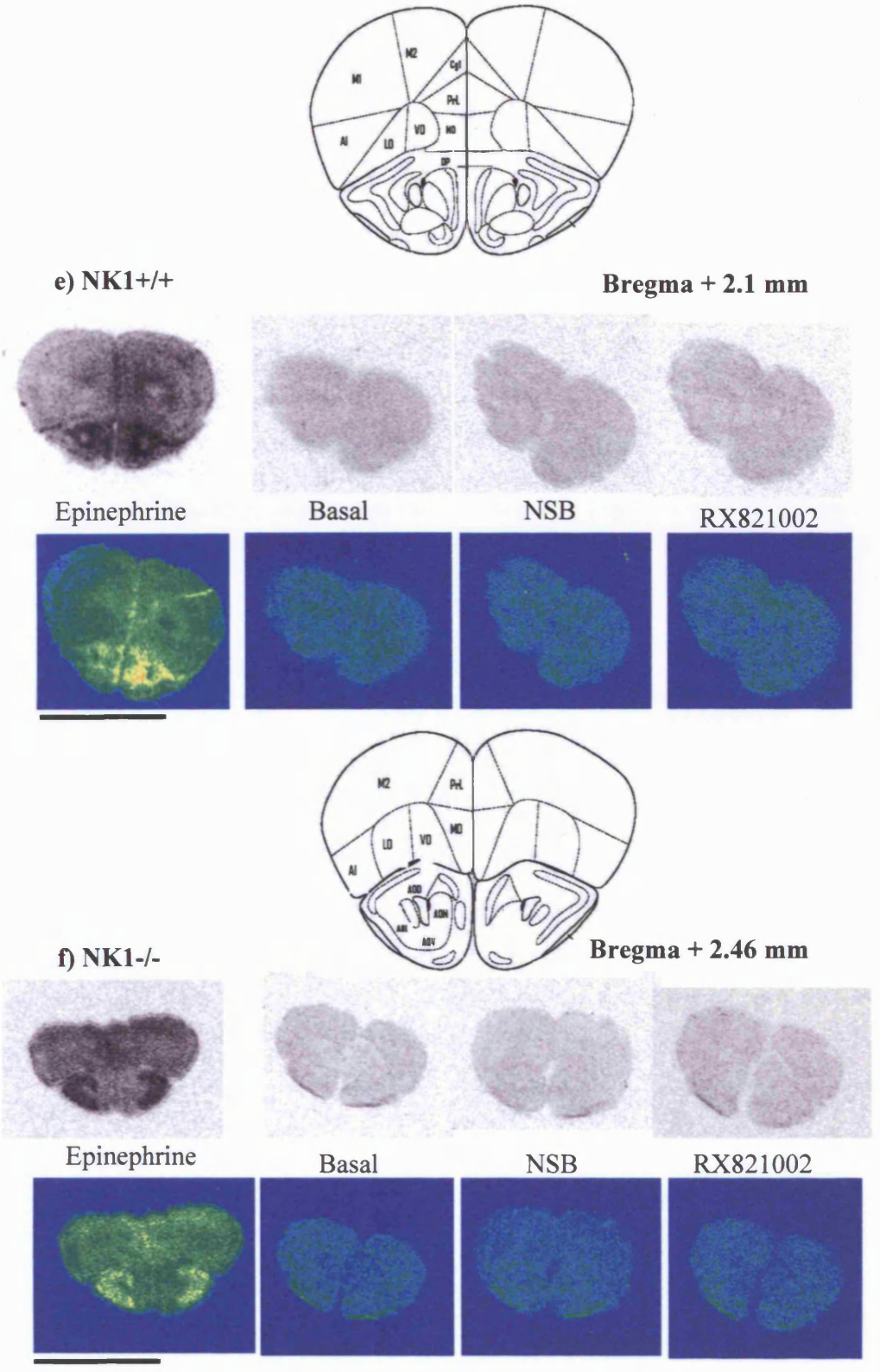


FIGURE 6.8e-f: Representative autoradiographs of α_2-adrenoceptor mediated increase in [^{35}S]GTPγS binding to brain sections from e) NK1+/+ and f) NK1-/- mice at the level of the frontal cortex. Coronal (15µm) sections were labeled by [^{35}S]GTPγS (0.05 nM), without (basal) or with 100µM epinephrine, at the level of the FCtx. Nonspecific (NSB) labeling was obtained from adjacent sections exposed to 100 µM epinephrine plus 100 µM RX821002. n = 8. Scale = 2500 µm.

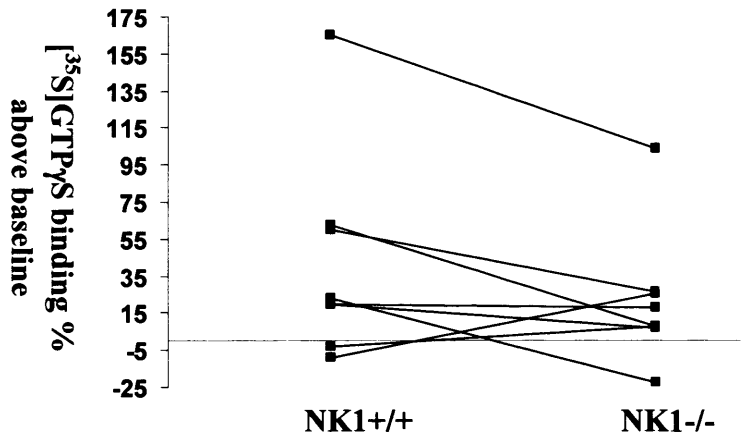


FIGURE 6.9: Line graphs of different autoradiographic films depicting the relationship of [^{35}S]GTPγS binding in the LC between NK1+/+ and NK1-/- mice. In the majority of autoradiographs [^{35}S]GTPγS binding was lower in NK1-/- mice.

[^{35}S]GTPγS binding (% over baseline)		
	LC	**FCtx (M2)**
NK1+/+	42.07 ± 19.74	49.76 ± 17.31
NK1-/-	21.55 ± 12.95	61.23 ± 21.71

TABLE 6.3: Adrenaline-stimulated [^{35}S]GTPγS binding to the locus coeruleus and frontal cortex of NK1+/+ and NK1-/- mice.

6.4 DISCUSSION

6.4.1 Regional binding of [^{3}H]RX821002 and [^{3}H]rauwolscine

Autoradiographic analysis of [^{3}H]RX821002 binding revealed extensive binding of this radioligand throughout the brain of both NK1+/+ and NK1-/- mice. No difference in binding density in the brain regions analysed was found between NK1+/+ and NK1-/- mice. [^{3}H]Rauwolscine demonstrated a more restricted regional pattern of binding throughout the brain of NK1+/+ and NK1-/- mice, again, no difference in binding density was found between NK1+/+ and NK1-/- mice. The regional binding of [^{3}H]RX821002 and [^{3}H]rauwolscine in the brain of NK1+/+ and NK1-/- mice resembles that found by Holmberg *et al.*, (Holmberg *et al.*, 2003) in inbred FVB/N mice and in the rat (Hudson *et al.*, 1992). Regional [^{3}H]RX821002 binding was analysed in limbic brain regions receiving a dense noradrenergic input from the LC.

6.4.1.1 Amygdala

Binding in the amygdala was detectable only with [^{3}H]RX821002, implying the predominance of the α_{2a}-adrenoceptor subtype in this brain region. However, no difference in α_{2a}-adrenoceptor binding density was found between NK1+/+ and NK1-/- mice. Immunohistochemical (Rosin *et al.*, 1996; Talley *et al.*, 1996) and autoradiographic studies have found both the α_{2a} and α_{2c}-adrenoceptor subtypes in the amygdala of the rat (Boyajian *et al.*, 1987) and mouse (Holmberg *et al.*, 2003). *In situ* hybridization studies report moderate α_{2a}-adrenoceptor labelling and light α_{2c}-adrenoceptor labelling in the amygdaloid complex of the rat (Scheinin, 1994) and mouse (Wang *et al.*, 1996). The lack of detectable binding of the α_{2c}-adrenoceptor in the amygdala in these studies is unexpected, therefore. The present studies used the same concentration of [^{3}H]RX821002 (1.0 nM) and [^{3}H]rauwolscine (0.4 nM) as Holmberg *et al.*, (2003), who did find α_{2c}-adrenoceptor labelling in the amygdala. Therefore, it is possible that the lack of detectable α_{2c}-adrenoceptor labelling in the amygdala is attributable to film exposure time.

The amygdala is an essential structure for fear processing (for review see: (LeDoux, 1998), formation of fear memories (Maren, 1999; Nader *et al.*, 2000) and mediating learned fear (Walker & Davis, 1997). The moderate amount of [^{3}H]RX821002 binding to

α_2-adrenoceptors in the amygdala supports an important role for NA release in this brain region, in mediating various responses to stressful stimuli through the amygdala (for review see: (Tanaka *et al.*, 2000)) .

6.4.1.2 Bed nucleus of the stria terminalis

Whereas the amygdala is important for learned fear, the BNST plays an important role in mediating unlearned fear (anxiety-like) behaviours e.g. startle (Lee & Davis, 1997; Davis *et al.*, 1997; Gewirtz *et al.*, 1998; Schweimer *et al.*, 2005).The highest concentration of NA in the brain is found within the BNST (Kilts and Anderson., 1986). Stress causes an increase in NA efflux in the BNST of rats (Delfs *et al.*, 2000; Fuentealba *et al.*, 2000; Wang *et al.*, 2001). In the BNST, NA exerts a tonic inhibitory action, regulated via α_2-adrenoceptors (Forray *et al.*, 1999). This is supported by the high amount of α_2-adrenoceptors found in the BNST in the present studies.

6.4.1.3 Cerebellum

Both the granule cell layer and molecular layer of the cerebellar cortex were measured. However, [^{3}H]RX821002 binding was higher in the granule cell layer compared with the molecular layer of the CBM. Again, [^{3}H]rauwolscine binding was not above film background. Both imunohistochemistry (Aoki *et al.*, 1994; Rosin *et al.*, 1996; Talley *et al.*, 1996) and *in situ* hybridisation (Nicholas *et al.*, 1993; Scheinin *et al.*, 1994; Wang *et al.*, 1996; Winzer-Serhan *et al.*, 1997b; Winzer-Serhan *et al.*, 1997a) studies have demonstrated the localisation of both α_{2a} and α_{2c}-adrenoceptors in the CBM.

Cerebellar NA is important for the adaptive ability to coordinate locomotor tasks e.g. the irregular rod runway paradigm, a novel motor task (Watson & McElligott, 1984; Bickford *et al.*, 1992). In the CBM, as in all brain regions, NA acts as a neuromodulator, augmenting the actions of neurotransmitters such as GABA (Bickford, 1995). The presence of α_2-adrenoceptors in this brain region, therefore, is expected.

6.4.1.4 Hippocampus

The hippocampus receives intense noradrenergic innervation. The highest binding in the hippocampus was found with [^{3}H]RX821002, supporting the predominance of the α_{2a}-adrenoceptor subtype. This is in agreement with that found by (Holmberg *et al.*, 2003). [^{3}H]Rauwolscine binding was also found in the CA1 area of the hippocampus, supporting a study demonstrating that both these subtypes modulate release of NA in this brain region (Trendelenburg *et al.*, 2001).

6.4.1.5 Locus coeruleus

Only [^{3}H]RX821002 binding was found in the LC. The lack of expression of α_{2c}-adrenoceptors in the LC demonstrated by a lack of [^{3}H]rauwolscine binding, was also found by (Holmberg *et al.*, 2003). The α_{2a}-adrenoceptor is believed to be the predominant subtype in the rodent brainstem as demonstrated by immunohistochemical (Rosin *et al.*, 1993) and *in situ* hybridization studies (Scheinin, 1994). The role played by LC-located α_{2a}-adrenoceptors in mediating the hypertensive, sedative and antinociceptive actions of α_2-adrenoceptor agonists, such as clonidine, have been well demonstrated (De Sarro *et al.*, 1987; Scheinin & Schwinn, 1992; Hunter *et al.*, 1997; Altman *et al.*, 1999). Although not demonstrated here, the presence of α_{2c}-adrenoceptors located on LC noradrenergic neurones cannot be excluded. Particularly as these two subtypes both contribute to the feedback regulation of NA release (Hein *et al.*, 1999).

6.4.1.6 Lateral septum

Only [^{3}H]RX821002 binding was detected in the lateral septum, suggesting the predominance of the α_{2a}-adrenoceptor subtype in this brain region. Dense labelling of α_{2a}-adrenoceptors in the rat septal area has been found using immunohistochemistry (Talley *et al.*, 1996). *In situ* hybridization studies show the presence of a large amount of mRNA for the α_{2a}-adrenoceptor in the septum of mouse (Wang *et al.*, 1996) and rat (Nicholas, 1993; Scheinin, 1994) brains. The lack of [^{3}H]rauwolscine binding, and hence α_{2c}-adrenoceptors, is in line with the majority of *in situ* hybridization studies from other laboratories (Scheinin, 1994; Wang *et al.*, 1996). The septum acts as a relay station, integrating the limbic telencephalon (forebrain) with the hypothalamus and the brainstem

(hindbrain) (Antonopoulos *et al.*, 2004). The lateral septum modulates descending limbic cortical pathways to diencephalic areas. NA inhibits lateral septum neuronal activity by activating α_2-adrenoceptors (Liu & Alreja, 1998).The dense binding of [^{3}H]RX821002 in this brain area is not surprising.

6.4.1.7 Frontal cortex

Only [^{3}H]RX821002 binding was observable in NK1+/+ and NK1-/- mice, supporting the predominance of the α_{2a}-adrenoceptor in the cortex. 90 % of cortical adrenoceptors are of the α_{2a} subtype, as determined using rat cortical homogenates (Uhlen *et al.*, 1992). Since noradrenergic neurotoxins e.g. DSP-4 do not effect radioligand binding (Heal *et al.*, 1993), the majority of cortical α_2-adrenoceptors are post-synaptic, and of the α_{2a}-adrenoceptor subtype (Heal *et al.*, 1995). All of the frontal cortex noradrenergic projections arise from the LC (Foote *et al.*, 1980a; Loughlin *et al.*, 1986b; Loughlin *et al.*, 1986a; Loughlin *et al.*, 1982). NA enhances cognitive processing by the prefrontal cortex through actions at post-junctional α_{2a}-adrenoceptors: (for review see: (Arnsten, 1997)). α_{2a}-Adrenoceptor agonists e.g. guanfacine, have cognitive-enhancing abilities, as demonstrated by the improved performance of aged monkeys in the delayed response performance (Arnsten, 1997), through actions at post-junctional α_{2a}-adrenoceptors. α_{2a}-Adrenoceptor agonists are particularly effective at enhancing working memory performance under distracting conditions (Arnsten & Contant, 1992). The presence of [^{3}H]RX821002 binding in these studies supports the actions of NA through α_{2a}-adrenoceptors in the frontal cortex.

6.4.1.8 Olfactory bulb

In the olfactory bulbs of both NK1+/+ and NK1-/- mice, a higher number of binding sites was recognised with [^{3}H]RX821002 than [^{3}H]rauwolscine, further supporting the predominance of α_{2a}-adrenoceptors. The low density of α_{2c}-adrenoceptor in the olfactory bulb is in agreement with that found by (Boyajian *et al.*, 1987) and (Holmberg *et al.*, 2003). However, both α_{2a}-adrenoceptor and α_{2c}-adrenoceptor mRNA is found in the olfactory nuclei (Wang *et al.*, 1996). NA innervation of the olfactory bulb is exclusively from the LC (McLean *et al.*, 1989), where it is thought to be important for olfactory learning. The LC input to the olfactory bulb is crucial for early olfactory learning, as LC lesions with 6-hydroxydopamine impair acquisition of conditioned odour preferences in

the newborn rat (Sullivan *et al.*, 1994). The role of NA in the olfactory bulb, as elsewhere, is believed to be modulatory, increasing the 'signal-to-noise' ratio in neuronal activation.

6.4.1.9 Periaqueductal grey

Only binding with [^{3}H]RX821002 was observed in the PAG, indicating an absence of α_{2c}-adrenoceptors in this brain region. Immunohistochemistry revealed only a sparse distribution of the α_{2c}-adrenoceptor subtype in this brain region (Rosin, 1996). *In situ* hybridization studies demonstrate only light labelling of mRNA for the α_{2c}-adrenoceptor in the PAG (Nicholas *et al.*, 1993). So, the apparent lack of [^{3}H]rauwolscine binding in these studies is not surprising if the α_{2c}-adrenoceptor is present in only small amounts. Noradrenergic neurones projecting to the PAG are thought to be important for coordinating the autonomic and motor behavioural response to stress, as well as mediating fear and panic reactions, (for review see: (Schenberg *et al.*, 2001)). A reciprocal, monosynaptic projection links the ventrolateral PAG to extranuclear dendrites of noradrenergic LC neurones. This monosynaptic pathway may partly mediate analgesia, reduced responsiveness to external stimuli, and decreased excitability of somatic motoneurones (Bajic *et al.*, 2000). Both the LC and central nucleus of the amygdala innervate the PAG (see: (Sullivan *et al.*, 1999)). The input from the amygdala is important for modulating freezing fear behaviour and pain (da Costa Gomez & Behbehani, 1995; Manning, 1998; Misslin, 2003; Oliveira *et al.*, 2004). The binding of [^{3}H]RX821002 to α_2-adrenoceptors in these brain regions is, therefore, not surprising (Redmond, Jr. & Huang, 1979).

6.4.1.10 Striatum

In these studies, binding of both [^{3}H]rauwolscine and [^{3}H]RX821002 were detected in the striatum. A high density of the α_{2c}-preferring radioligand [^{3}H]rauwolscine in the striatum has also been described previously by Holmberg *et al.*, in the mouse (Holmberg *et al.*, 2003). Both *in situ* hybridization and immunohistochemical studies report high expression of mRNA for the α_{2c}-adrenoceptor subtype in the striatum of the rat (Boyajian *et al.*, 1987; Nicholas *et al.*, 1993; Scheinin *et al.*, 1994; Rosin *et al.*, 1996; Winzer-Serhan *et al.*, 1997b; Uhlen *et al.*, 1997) and mouse (Wang *et al.*, 1996). However, whether or not there is any appreciable noradrenergic innervation of the striatum has been questioned; hitherto

only a modest noradrenergic input to the striatum has been found (Swanson & Cowan, 1979). The dense expression of the α_{2c}-adrenoceptor subtype in the striatum, an area of high dopaminergic innervation (Boyajian *et al.*, 1987; Rosin *et al.*, 1996), has, therefore, prompted the proposal that it may be DA that activates these receptors (see: (Holmberg *et al.*, 2003)). Furthermore, α_{2c}-adrenoceptor knock-out mice show a decrease in homovanillinc acid (a DA metabolite) (Sallinen *et al.*, 1997), demonstrating a role played by these receptors in DA turnover. α_{2c}-Adrenoceptors have an inter-neuronal localisation in GABAergic medium-sized spiny projection neurons in the rat striatum, where they may modulate both the direct and indirect pathway of the basal ganglia (Holmberg *et al.*, 1999).

The striatum plays a major role in coordinating movement and producing pre-programmed movements (engrams). The localisation of the α_{2c}-adrenoceptor subtype in this brain area, supports the role played by α_{2c}-adrenoceptors in motor performance. The role played by α_{2c}-adrenoceptors is demonstrated by the locomotor stimulatory activity of the indirect DA agonist, *d*-amphetamine. Mice over-expressing this receptor show a blunted increase in locomotion (Sallinen *et al.*, 1998), suggesting that α_{2c}-adrenoceptors have an inhibitory influence on the hyperlocomotion elicited by *d*-amphetamine.

6.4.2 Choice of radiolabel

[^{3}H]RX821002 has the advantage that, as an antagonist, it labels the entire population of α_2-adrenoceptors whereas agonists label the high affinity state of the receptor preferentially. The current results confirm the contention of Langin *et al.*, (1989) and Hudson *et al.*, (1992), that [^{3}H]RX821002 is an excellent radioligand for the receptor binding studies of α_2-adrenoceptors as it displays high affinity and very low non-specific binding.

[^{3}H]RX821002 was chosen for these studies for a number of reasons. After tritiation RX821002 retains its pharmacological selectivity and consequently makes it an excellent radioligand for use in receptor binding studies (see: (Clarke & Harris, 2002)). [^{3}H]RX821002 has the lowest percentage of non-specific binding compared with other α_2-adrenoceptor antagonists such as [^{3}H]yohimbine, [^{3}H]idazoxan and [^{3}H]rauwolscine, which are also used for radioligand binding studies (Langin *et al.*, 1989). Although it is

generally thought of as an α_2-adrenoceptor non-subtype selective antagonist, studies have suggested RX821002 shows a high selectivity for the α_{2a}-adrenoceptor subtype (Langin *et al.*, 1989; Uhlen *et al.*, 1998). In potassium phosphate buffer, as used here, [^{3}H]RX821002 has a moderately higher affinity for the human α_{2a}-adrenoceptor subtype than for α_{2c} or α_{2b} (Halme *et al.*, 1995). Equilibrium dissociation constants (K_d) for [^{3}H]RX821002 in potassium phosphate buffer were, 0.48 nM for the α_{2a}-adrenoceptor, 3.07 nM for α_{2b}-adrenoceptor and 0.63 nM for α_{2c}-adrenoceptor.

To ensure labelling of the α_{2a}-adrenoceptor, [^{3}H]rauwolscine was included in these studies. Rauwolscine preferentially labels the α_{2c}-adrenoceptor subtype in cells transfected with human (K_i 1.0), mouse (K_i 0.8) or rat (K_d 0.8) α_2-adrenoceptor subtype genes (Harrison *et al.*, 1991; Link *et al.*, 1992; Marjamaki *et al.*, 1993). Due to its low affinity for rat α_{2a}-adrenoceptors (K_d 34 nM), compared with the rat α_{2c}-adrenoceptor (K_d 1.6 nM) (Uhlen *et al.*, 1998) low concentrations of rauwolscine can, therefore, be used to differentiate between α_{2a} and α_{2c}-adrenoceptors in autoradiographic studies (Boyajian *et al.*, 1987; Boyajian & Leslie, 1987). It was, therefore, believed that subtraction of the [^{3}H]rauwolscine binding density, from that of the [^{3}H]RX821002 binding density, would give a good indication of the α_{2a}-adrenoceptor population. [^{3}H]rauwolscine binding was limited to the striatum, CA1 area of the hippocampus and accumbens, only, supporting previous reports of the predominance of the α_{2a}-adrenoceptor subtype in the CNS.

6.4.3 Caveats

These studies using [^{3}H]RX821002 and [^{3}H]rauwolscine demonstrate no differences in the α_2-adrenoceptor population between NK1-/- and NK1+/+ mice. It is possible that the autoradiographic technique is not sensitive enough to detect small changes in α_2-adrenoceptor density that may account for the lack of response to RX21002 in NK1-/- mice. Alternatively, the lack of response to RX821002 demonstrated in NK1-/- mice could be attributed to a mechanism downstream of the receptor recognition site. α_2-Adrenoceptors are G protein-coupled receptors. Therefore, these studies progressed to looking at the first step in the receptor transduction sequence: the ability of α_2-adrenoceptors to bind GTP.

6.4.4 *[^{35}S]GTPγS analysis of the functional activity of α_2-adrenoceptors in NK1+/+ and NK1-/- mice*

Although no difference in [^{3}H]RX821002 and [^{3}H]rauwolscine binding density was found between genotypes, this does not exclude the possibility that the difference in basal NA efflux could be due to a functional uncoupling of the α_2-adrenoceptor from its G protein or second messengers downstream of the recognition site. Uncoupling of the α_2-adrenoceptor from the G protein can occur in the absence of receptor downregulation (O'connor *et al.*, 2005).

However, no difference in adrenaline-stimulated [^{35}S]GTPγS binding was observed between NK1-/- and NK1+/+ mice in either the LC or frontal cortex (area M2). The current studies have, therefore, been unable to identify molecular correlates for the increase in basal NA efflux observed in anaesthetised NK1-/- mice compared to their NK1+/+ counterparts. Further investigations into the downstream signalling pathway of the α_2-adrenoceptor are required to determine if NK1 receptor disruption is a cause or a consequence of the increase in basal NA efflux in NK1-/- mice, and the lack of response to an α_2-adrenoceptor antagonist observed in these studies.

6.4.5 *Future candidates to investigate downstream of the α_2-adrenoceptor*

6.4.5.1 Adenylate cyclase activity

Second messenger signalling of α_2-adrenoceptors is mediated via adenylate cyclase (AC). Activation of these receptors typically decreases AC activity, which decreases cAMP production. PKC-stimulated activity of adenylate cyclase type2 (AC2) is inhibited by G protein-coupled receptors that activate G_o proteins, but potentiated by G_i proteins. This difference between G_o and G_i proteins implicates a potential switch in the cAMP-PKA signalling pathway, by $G_{i/o}$-coupled receptors, depending on which G protein is available for activation (Nasman *et al.*, 2002). The molecular switch may be different in the two genotypes; A consequence of disrupting the NK1 receptor may be that the NK1-/- mice stimulate AC through G_i leading to increased release of Ca^{2+} from internal stores. Future studies could investigate the rate of cAMP synthesis in NK1+/+ and NK1-/- mice.

6.4.5.2 G protein linked inwardly rectifying potassium channels

Activation of α_2-adrenoceptors results in the opening of G protein linked inwardly rectifying K^+ channels (GIRKS) and closure of Ca^{2+} channels, resulting in a decrease in noradrenergic cell firing. Activation of NK1 receptors by SP produces the opposite excitatory effect on noradrenergic cell bodies: closure of GIRKs and opening cation channels (Shen & North, 1992). The LC has among the highest density of NK1 receptors in the rat brain (Mantyh *et al.*, 1989) and nerve terminals, showing SP-like immunoreactivity, form axodendritic synapses with catecholaminergic neurones (Pickel *et al.*, 1979). The SP input to the LC is likely to arise from the nucleus reticularis paragigantocellularis (Dean *et al.*, 1993), or the nearby parabrachial nucleus (see: (Shen & North, 1992)). Disruption of the NK1+/+ receptor may, therefore, lead to a compensatory adaptation in how the noradrenergic cells maintain their excitability. For example, the activity of LC located GIRKS may be decreased in NK1-/- mice, which could lead to an increased rate of LC noradrenergic cell firing. Further studies are required to confirm this.

6.4.5.3 Regulators of G protein signalling

Regulators of G protein signalling (RGSs) negatively regulate G protein signalling. For example, RGS4, a G_α-interacting protein (GAIP), selectively enhances α_{2a}-adrenoceptor stimulation, of the intrinsic GTPase activity of the G protein α subunit ($G\alpha_{o1}$ and $G\alpha_{i2}$ subtypes) (Cavalli *et al.*, 2000), thereby terminating signalling through the inhibitory α_2-adrenoceptor. A developmental up regulation of RGS4 in NK1-/- mice, could result in the increase in basal NA efflux, as RGS4 GTPase activation facilitates attenuation of the functional output of G protein-coupled signalling. Further studies, for example Western blot protein analysis, would be useful to determine if the amount of RGS4 protein is altered in NK1-/- mice.

6.4.5.4 G protein-coupled receptor dimerization

μ opioid receptors are also G protein-coupled receptors, and couple to similar second messenger systems as the α_{2a}-adrenoceptor (Jordan & Devi, 1998). There are few areas where expression of the NK1 receptor, μ opioid receptor and α_{2a}-adrenoceptor receptor overlap: one exception is the LC. As well as being strongly implicated in stress and arousal, the LC is also involved in the development of opiate dependence (Nestler *et al.*, 1999).

Evidence supporting a functional interaction between the μ opioid and α_{2a}-adrenoceptor is provided by mice lacking the α_{2a}-adrenoceptor (Stone *et al.*, 1997). In these animals the analgesic potency to spinally administered morphine is decreased (Stone *et al.*, 1997). G protein-coupled receptor dimerization has been shown to be necessary for the functional activity of certain G proteins (Marshall *et al.*, 1999; Nelson *et al.*, 2001). Furthermore, in certain situations these interactions have been shown to result in changes in the activity of G protein-coupled receptors. For example, changes in ligand affinity, efficacy, trafficking, and/or desensitization (Gomes *et al.*, 2001; Angers *et al.*, 2002; Pfeiffer *et al.*, 2003). The physical interaction between α_{2a}-adrenoceptors and μ opioid receptors plays an important role in modulating their signalling, which could be brought about, at least in part, by the agonist-induced changes in receptor conformation and / or association (Jordan *et al.*, 2003). Activation of either the α_{2a}-adrenoceptor, or the μ opioid receptor, results in an increase in the extent of signalling through the μ-α2a dimer, whereas activation of both receptors leads to a decrease in receptor signalling (Jordan *et al.*, 2003). One possible hypothesis, therefore, for the increase in basal NA efflux in halothane-anaesthetised NK1-/- mice, could be an increase in signalling through the μ-α_{2a} receptor complex by both endogenous ligands, resulting from disruption of the NK1 receptor. This could lead to a decrease in the extent of signalling and consequently an increase in NA release.

6.4.6 Methodological considerations

6.4.6.1 Immunohistochemistry and Western blot protein analysis

The major drawback associated with the investigation of the α_{2a}-adrenoceptor subtype using IHC and Western blot protein analysis was the lack of a suitable, commercially-available, antibody, and the small amount of α_{2a}-adrenoceptor protein in the LC region.

Although preliminary IHC results of the α_{2a}-adrenoceptor in the LC region suggested a difference in the density of the receptor in the two genotypes, the results were not clear enough to quantify. It was hoped that Western blot protein analysis, which is believed to be a more sensitive technique than IHC, would provide quantifiable results. One of the major problems associated with the Western blot experiments was the lack of specificity of the antibody. This resulted in a high level of background making analysis of the protein bands difficult. This was resolved by switching the incubating buffer from semi-skimmed milk to bovine serum albumin, as it was believed the antibody was reacting with the semi-skimmed milk. The normal amount of protein used for Western blot analysis is 10 μg. In order for a signal to be found with the α_{2a}-adrenoceptor antibody, in the LC, it was necessary to increase the amount of protein to 30 μg, suggesting a low amount of receptor protein in this area of the brain.

Without using techniques such as a tissue punch to dissect out the LC, it is hard to obtain an accurate dissection of this brain area. So it is possible that areas around the LC were included in the dissection. However, the α_{2b}-adrenoceptor subtype is found only in the thalamus (Nicholas *et al.*, 1993; Scheinin, 1994), and the localisation of the α_{2c}-adrenoceptor-preferring radioligand [^{3}H]rauwolscine in the striatum demonstrates that the α_{2a}-adrenoceptor subtype is the most likely receptor located in the area of the LC.

6.4.6.2 Adrenaline-stimulated [^{35}S]GTPγS binding

Available evidence supports the expression of α_2-adrenoceptors, α_1-adrenoceptors and β-adrenoceptors in the same regions of the CNS (Nicholas *et al.*, 1996). It is possible

that adrenaline-stimulated [^{35}S]GTPγS binding was occurring at α_1-adrenoceptors and β-adrenoceptors. However, regions expressing high levels of α_1-adrenoceptors and β-adrenoceptors, such as cerebellum and thalamus (Rainbow *et al.*, 1984; Jones *et al.*, 1985), did not demonstrate high levels of adrenaline-stimulated [^{35}S]GTPγS activity. Furthermore, α_1-adrenoceptors and β-adrenoceptors are coupled to pertussis toxin insensitive G_q and G_s G proteins, respectively. It is believed that receptors that stimulate [^{35}S]GTPγS binding are coupled to $G_{i/o}$ G proteins, only. Therefore, in these studies, most of the adrenaline-stimulated binding is likely to be due to the α_2-adrenoceptor, possibly the α_{2a}-adrenoceptor subtype, as this is the predominant α_2-adrenoceptor expressed in the CNS (Talley *et al.*, 1996; Rosin *et al.*, 1996). This is supported by the radioligand binding study with [^{3}H]RX821002 and [^{3}H]rauwolscine, showing negligible [^{3}H]rauwolscine binding to the α_{2c}-adrenoceptor in the LC or frontal cortex (M2), the brain areas of particular interest in these studies.

Tissue sections from NK1+/+ and NK1-/- mice were processed together on films. It is interesting, therefore, to note that in 5 out of 8 films of the LC area, NK1-/- mice demonstrated *lower* percentage above baseline adrenaline-stimulated [^{35}S]GTPγS binding compared to NK1+/+ mice. It is, therefore, tempting to speculate that by increasing the n number in this study, a decrease in the functional activity of the α_2-adrenoceptors in NK1-/- mice would be demonstrated. 'Power Analysis' can be used to calculate the required sample size of simple data. Using 'Power Analysis' to calculate the required n number for the adrenaline-stimulated [^{35}S]GTPγS binding assay, we find that when n = 8, there is only a 12.8 % chance that the experiment would detect a difference in the functional activity of α_2-adrenoceptors between NK1+/+ and NK1-/- mice. A 90 % chance that the experiment would detect a difference in the functional activity of the α_2-adrenoceptors between the two genotypes would require n = 108.2, a very substantial and expensive experiment. It is possible that the biological decrease in the functioning of the α_2-adrenoceptor in NK1-/- mice is too small to be detected statistically. Increasing the n number could result in significance being reached, alternatively, resources could be wasted.

6.4.7 Summary and conclusions

The studies presented in this chapter were unable to find a definitive molecular correlate to explain the higher basal NA efflux and lack of response to an α_2-adrenoceptor antagonist in NK1-/- mice. Future studies should, therefore, be aimed at investigating targets downstream of the G protein-coupled receptor as a possible cause of the increased NA efflux in NK1-/- mice.

These studies were unable to identify a difference in the molecular substrates responsible for the control of synthesis, reuptake and release of NA between NK1+/+ and NK1-/- mice. The following chapter, therefore, describes an experiment aimed at determining whether a difference in the content of noradrenergic vesicles (which contain the releasable pool of transmitter) could underlie the difference in NA efflux between NK1+/+ and NK1-/- mice.

CHAPTER 7

ANALYSIS OF THE CONCENTRATION OF NORADRENALINE IN NEURONAL STORAGE VESICLES OF NK1+/+ AND NK1 -/- MOUSE CORTEX

7 ANALYSIS OF THE CONCENTRATION OF NORADRENALINE IN NEURONAL STORAGE VESICLES OF NK1+/+ AND NK1 -/- MOUSE CORTEX

7.1 INTRODUCTION

Experiments described in this chapter investigated whether the increase in NA efflux in anaesthetised NK1-/- mice is due to an increase in the releasable pool of NA in the terminal storage vesicles. Although α_2-adrenoceptors control release of NA, other factors including size of the releasable pool and intraterminal Ca^{2+} concentration, which is influenced indirectly by α_2-adrenoceptors, should be explored.

7.1.1 Background

NA is stored in vesicles which can be viewed under the electron microscope and isolated by differential centrifugation (Fillenz & Stanford, 1981). The vesicular NA in sympathetic nerve terminals (noradrenergic) is divided into two functional pools: a small release pool and a larger storage pool which is not easily mobilised for release (Swedin, 1972; Glowinski, 1973). These vesicles take up and store NA from the neuronal cytoplasm. The vesicular content can be increased by a range of treatments, such as monoamine oxidase (MAO) inhibition, or injection of exogenous NA and depleted by intense physical stimulation (see: (Fillenz & Howe, 1971; Fillenz & Stanford, 1981)).

Early studies of transmitter release relied on measuring its concentration in the perfusate of a stimulated, perfused sympathetic nerve / end-organ preparation. Brown and colleagues (Brown, 1965) pioneered analysis of perfusate during and after stimulation of noradrenergic neurones using the cat splenic nerve and spleen. Brown, demonstrated that, in addition to NA, the perfusate collected from the spleen after splenic nerve stimulation contained DβH but not cytoplasmic proteins e.g. lactate dehydrogenase and tyrosine hydroxylase (Fillenz, 1990a). The presence of DβH, which is found only in NA storage vesicles, confirmed that NA was released from vesicle-bound packets of transmitter contained within the terminal vesicles via exocytosis.

The terminal vesicles were isolated using differential centrifugation. This technique has been used in the past, to characterise the subcellular distribution of NA in sympathetic nerve terminals (Bisby & Fillenz, 1971). The technique has helped to identify the relative roles of reuptake and synthesis of transmitter in the maintenance of transmitter vesicular stores (Fillenz & Howe, 1975). Differential centrifugation has also confirmed that vesicles have a limited storage capacity for NA, that the NA content varies from organ to organ and that the degree of unsaturation determines how much additional NA can be taken up into the vesicles (Fillenz & Howe, 1971). Studies by Fillenz *et al.*, have also shown that increasing the activity of noradrenergic neurones *in vivo* leads to parallel changes in the NA content of storage vesicles and the NA release rate (Fillenz & Stanford, 1978). Using differential centrifugation it is also possible to measure vesicular NA concentration in brain-derived synaptosomes. Synaptosomes are essentially 'pinched' off nerve terminals.

It was, therefore, thought possible that an increase in NA release in NK1-/- mice could be due to an increase in the size of the vesicular store of NA in cortical tissue. This was explored using differential centrifugation.

7.3 RESULTS

No difference in the amount of NA content expressed as fmol / mg protein was found between NK1+/+ and NK1-/- mice (Table 7.1 and Figure 7.1).

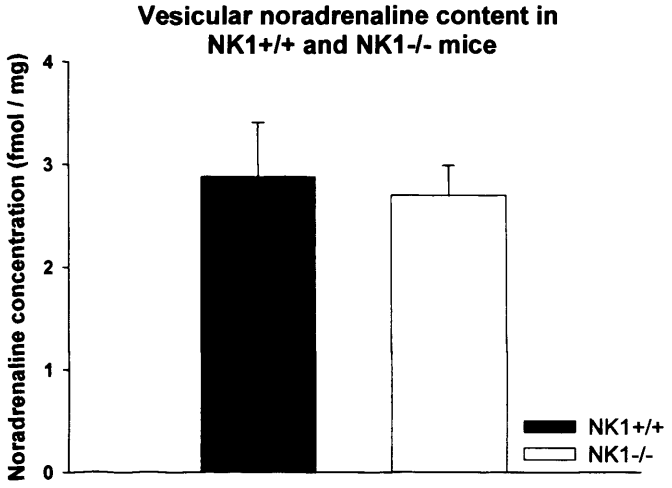


FIGURE 7.1: Noradrenaline concentration in cortical synaptosomes from NK1+/+ and NK1-/- mice. No difference in noradrenaline concentration was found between NK1+/+ and NK1-/- mice. Values are mean noradrenaline concentration expressed as fmol / mg ± s.e.m. n = 5.

	n	Average P_3 protein content mg / ml ± s.e.m.	Average S_4 NA content fmol / ml ± s.e.m.	Concentration of NA S_4/P_3 fmol / mg ± s.e.m.
NK1-/-	5	91.51 ± 8.53	237.64 ± 8.68	2.69 ± 0.3
NK1+/+	5	88.81 ± 4.49	252.4 ± 45.61	2.88 ± 0.53

TABLE 7.1: Noradrenaline content of microsomal pellet and final supernatant from the cortex of NK1+/+ and NK1-/- mice.

7.4 DISCUSSION

7.4.1 No difference in the vesicular noradrenaline content between NK1+/+ and NK1-/- mice

These studies show that there is no difference in vesicular NA content in synaptosomes isolated from cortical tissue in NK1+/+ and NK1-/- mice. Therefore, a difference in NA concentration in neuronal vesicles does not underlie the 4-5 fold difference in basal NA efflux observed in halothane-anaesthetised NK1+/+ and NK1-/- mice. The studies contained in this chapter do not rule out the possibility of an adaptive change in the firing-rate of noradrenergic neurones as a result of disrupting the NK1 receptor. Further studies are, therefore, required to investigate the firing-rate of LC-noradrenergic neurones. If firing-rate is unchanged between NK1+/+ and NK1-/- mice it is likely that the difference in the regulation of the noradrenergic system in NK1-/- lies at the level of the stimulus-secretion coupling. Preliminary evidence in this lab is beginning to support this hypothesis (Yan-unpublished observations; Section 7.4.2.2).

However, these studies do not exclude the possibility of an increased number of vesicles in the readily releasable pool near the 'active zone' in NK1-/- mice, or that vesicles may be more readily mobilised in NK1-/- mice. The readily releasable pool comprises those vesicles located near to the 'active zone', where vesicle fusion with the axolemma takes place and which is richly endowed in Ca^{2+} channels. The reserve pool comprises vesicles docked more remotely on the neuronal cytoskeleton (see: Stanford, 2001c). Alterations at various stages in the mechanism of vesicular release could account for the increase in basal NA efflux in NK1-/- mice (Figure 7.2).

7.4.2 Alternative possibilities

7.4.2.1 Alteration in the distribution of vesicles

Both large and small dense core vesicles store NA (Fillenz, 1990b). However, small dense core vesicles (SDCVs) are believed to contain most of the NA in nerve terminals (Bisby & Fillenz, 1971; Fillenz & Stanford, 1978). The amount of NA available for release may depend on the extent to which these vesicles are filled with transmitter,

which can vary from organ to organ (Fillenz & Stanford, 1981; Fillenz & Pollard, 1976). The release of NA relies on burst-firing of noradrenergic neurones.

7.4.2.2 Intracellular calcium

Stimulus-secretion coupling is dependent on the internal Ca^{2+} concentration of the neurone. Arrival of a nerve impulse results in the opening of Ca^{2+} channels. Intracellular Ca^{2+} release occurs via the endoplasmic reticulum and other sites (calcisomes) (for review see: (Siesjo, 1990)). The rise in intraterminal Ca^{2+} concentration triggers the fusion of vesicles with the nerve terminal membrane, and the release of NA via exocytosis. α_2-Adrenoceptors negatively regulate Ca^{2+} channels. The $\beta\gamma$ subunit dissociates from the trimeric ($\alpha\beta\gamma$) G protein, and binds to the Ca^{2+} channel, shifting the voltage sensitivity to more positive potentials, so that the channels do not open so readily during rapid membrane depolarisation. Activation of the NK1 receptor has the opposite effect on Ca^{2+} channels. It is possible, that a compensatory consequence of losing an excitatory input to the cell is that Ca^{2+} channels in NK1-/- mice are less sensitive to the depolarisation blockade caused by α_2-adrenoceptors. Preliminary evidence in this lab is beginning to support this proposal. This preliminary evidence demonstrates that NK1+/+ mice are less resistant to Ca^{2+} depletion in K^+-stimulated tissue compared with NK1-/- mice (Yan-unpublished observations).

7.4.2.3 Mobilisation of vesicles

The phosphorylation / dephosphorylation of Synapsin I (a vesicle bound protein), is controlled by kinase proteins, and, regulates the cycling of vesicles between the readily releasable vesicular pool and the more remote vesicular storage pool (Hilfiker *et al.*, 1999a). Stimuli which increase Ca^{2+}-dependent stimulus-secretion coupling induce phosphorylation of Synapsin I. Phosphorylation of synapsin induces dissociation of synapsin from the cytoskeleton and the vesicles, mobilising the vesicle to the release pool. A number of protein kinases target synapsins. However, one in particular, Ca^{2+} / calmodulin protein kinase II (CAM kinase II), is bound to vesicle membranes. It is believed that an influx of Ca^{2+} triggers the phosphorylation of synapsin by CAM kinase II (Figure 7.2). It is possible, therefore, that in NK1-/- mice synapsin is phosphorylated more readily by CAM kinase II.

7.4.2.4 Docking-priming of the vesicles

The docking, fusion and release of vesicles is believed to involve distinct interactions between proteins bound to the axolemma, vesicles and soluble cytoplasmic proteins (Figure 7.3). Synaptotagmin (Figure 7.2) a vesicular bound protein is widely believed to modulate the influence that Ca^{2+} has on the docking of vesicles to the axolemma (Littleton & Bellen, 1995). The NH_2-tail of synaptotagmin penetrates the vesicle, whereas, the COOH-tail extends into the cytoplasm. This tail binds Ca^{2+}, possibly allowing synaptotagmin to act as a 'Ca^{2+}' sensor. Synaptotagmin is also phosphorylated by CAM kinase II (Hilfiker *et al.*, 1999b), which can regulate neurotransmitter release. Therefore, in NK1-/- mice the sensitivity of synaptotagmin may be increased, so that NA vesicles are more readily docked and primed for exocytotic release (Figure 7.2).

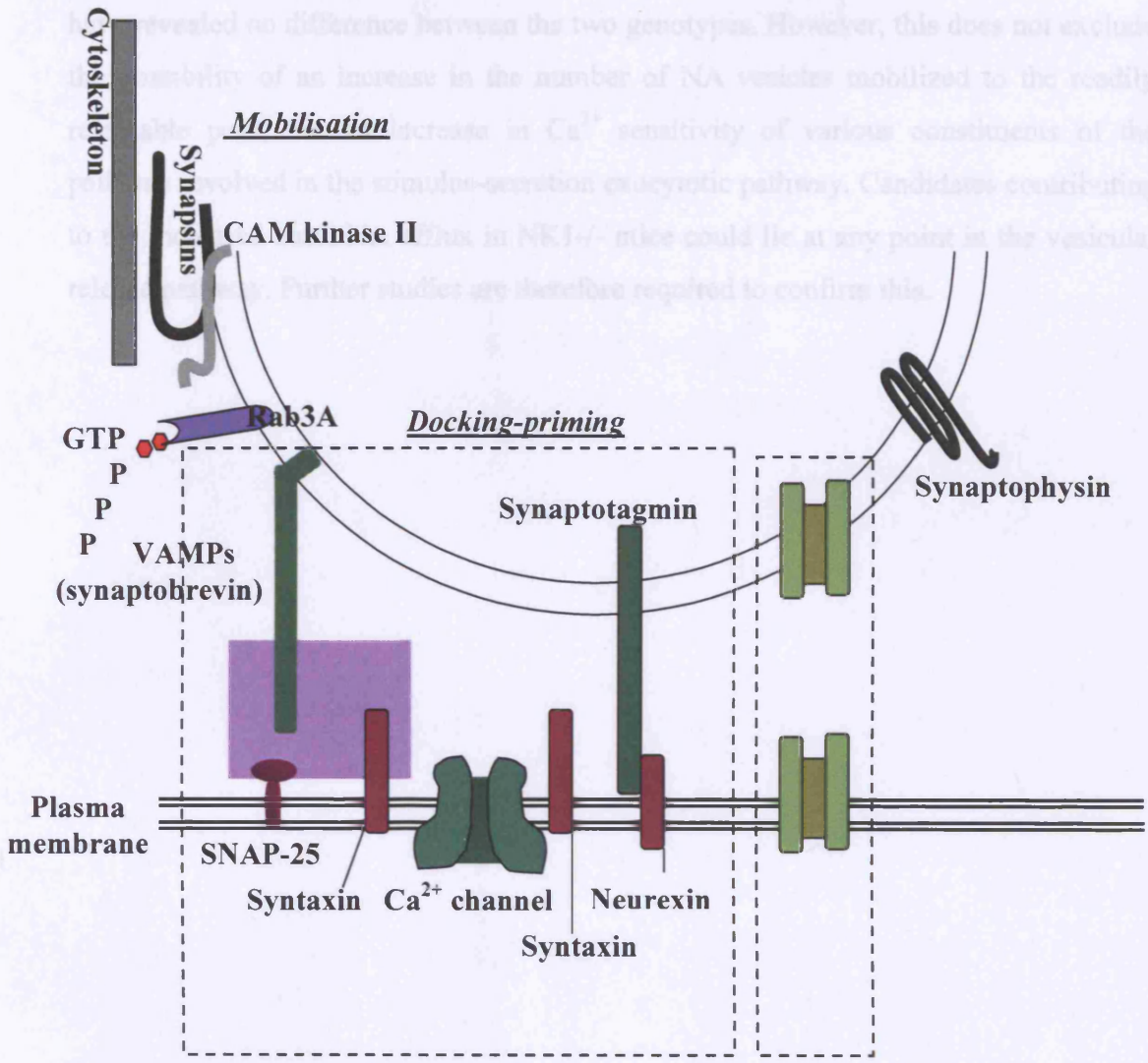


FIGURE 7.2: Diagrammatic representation of potential release-regulating sites that may be altered by genetic disruption of the NK1 receptor. 1) An increase in phosphorolylation of synapsin by CAM kinase II would lead to an increase in vesicular mobilisation in NK1-/- mice. 2) An increase in the Ca2+ sensitivity of synataptotagmin, or, an increase in internal Ca2+ concentration, would lead to an increase in the docking-priming of vesicles for release, in NK1-/- mice (Adapted from Kandel *et al.*, 1991). For a detailed review of all the factors involved in the excytotic cycle (See Benfanati *et al.*, 1999)

7.5 SUMMARY AND CONCLUSIONS

Investigations into the NA content of cortical NA vesicles in NK1+/+ and NK1-/- mice have revealed no difference between the two genotypes. However, this does not exclude the possibility of an increase in the number of NA vesicles mobilized to the readily releasable pool, nor an increase in Ca^{2+} sensitivity of various constituents of the pathway involved in the stimulus-secretion exocytotic pathway. Candidates contributing to the increased basal NA efflux in NK1-/- mice could lie at any point in the vesicular release pathway. Further studies are therefore required to confirm this.

CHAPTER 8

SUMMARY AND GENERAL DISCUSSION

8 SUMMARY AND GENERAL DISCUSSION

8.1 SUMMARY OF KEY FINDINGS

Initial microdialysis studies confirmed the earlier pilot findings that halothane-anaesthetised NK1-/- mice display a 2-5-fold higher basal NA efflux in their frontal cortex compared with their wildtype counterparts (Chapter 3; Fisher *et al.,* 2004; Stewart *et al.,* 2004; Herpfer *et al.,* 2005). These microdialysis studies further demonstrated that NA efflux in halothane-anaesthetised NK1+/+, but not NK1-/- mice, is increased following systemic administration of the α_2-adrenoceptor antagonist, RX821002 (0.3 mg / kg i.p.).

The second experiment described in Chapter 3 was designed to mirror that performed by Herpfer *et al.,* (2005). The studies of Herpfer *et al.,* (2005), focused on the role of terminal α_2-adrenoceptors in the control of NA efflux, by combining cortical infusion of RX821002 with systemic administration of desipramine (DMI). By combining cortical infusion of DMI with systemic administration of RX821002, the studies described here, provide extended information on the role of both terminal and somatodendritic α_2-adrenoceptors in the regulation of NA efflux in NK1+/+ and NK1-/- mice. By comparing the current findings, with those of Herpfer *et al.,* (2005), it appears that the regulatory influence of somatodendritic and terminal α_2-adrenoceptors, on NA efflux, becomes evident during situations which are presumed to alter the firing-rates of LC-noradrenergic cells and release of NA.

Cortical infusion of RX821002, resulted in the same incremental increase in NA efflux in both NK1+/+ and NK1-/- mice, demonstrating that terminal α_2-adrenoceptors tonically constrain cortical NA efflux in both genotypes (Herpfer *et al.*, 2005). Furthermore, cortical infusion of RX821002 augments the DMI induced increase in NA efflux in NK1+/+ mice, only, suggesting a dysregulation of the terminal α_2-adrenoceptor in NK1-/- mice. Systemic DMI, by increasing NA efflux, is likely to increase the autoregulatory activity of both terminal and somatodendritic adrenoceptors (See: Chapter 3). Therefore, in the studies of Herpfer *et al.,* (2005) cortical infusion of RX821002 revealed a difference in the regulation of terminal α_2-adrenoceptors at lower rates of LC-noradrenergic cell firing.

In contrast to the findings of Herpfer *et al.*, (2005), the studies reported here (Chapter 3) demonstrated no augmentation of the RX821002 (0.3 mg /kg i.p.) induced increase in NA efflux in NK1+/+ mice, following local infusion of DMI into the frontal cortex. This suggests a 'ceiling effect', following systemic administration of RX821002 in NK1+/+ mice, which constrains a further increase in NA efflux. Since local cortical infusion of RX821002, which antagonises only terminal α_2-adrenoceptors, results in the same incremental increase in NA efflux in both genotypes (Herpfer *et al.*, 2005), the difference in the tonic regulation of NA release, between NK1+/+ and NK1-/- mice, is likely to lie at the level of cell bodies in the LC. Therefore, in contrast to terminal α_2-adrenoceptors, the activity of which is revealed at lower LC firing-rates following systemic DMI, the difference in activity of somatodendritic α_2-adrenoceptors is revealed at higher rates of LC firing following systemic RX821002.

In the studies described here NA efflux in NK1-/- mice was not altered following drug challenge with either systemic RX821002, or local infusion of DMI, administered separately or in combination. The lack of effect of the α_2-adrenoceptor antagonist, RX821002, on NA efflux in NK1-/- mice, is consistent with the current hypothesis that genetic disruption of the NK1 receptor attenuates the activity of this autoregulatory receptor.

Systemic administration of DMI, by inhibiting reuptake through both somatodendritic and terminal NATs results in the same incremental increase in NA efflux in both genotypes (Herpfer *et al.*, 2005). By increasing endogenous noradrenergic tone at somatodendritic α_2-adrenoceptors it is likely that systemic DMI decreases the firing-rate of LC neurones (Mateo *et al.*, 1998; Svensson & Usdin, 1978; Invernizzi & Garattini, 2004) resulting in a blunted increase in NA efflux. Local cortical infusion of DMI is unlikely to change the firing-rate of LC-neurones. Therefore, it is possible that NA efflux following infusion of DMI may be so great in NK1-/- mice that the system, which is primed for maintenance of a higher basal noradrenergic activity, attempts to maintain a homeostatic level of NA by removal of NA through alternative transporters e.g. the dopamine transporters, organic cation transporters or via Uptake 2.

In contrast to NA efflux during continuous anaesthesia, there was no genotype dependent difference in basal NA efflux in freely-moving animals (Chapter 4). It is,

therefore, fortuitous that preliminary studies were conducted under anaesthesia, as the difference in regulation of the noradrenergic system between NK1+/+ and NK1-/- mice would otherwise have been overlooked. Furthermore, in contrast to the anaesthetic situation, freely-moving mice of both genotypes did not respond to 0.3 mg / kg i.p. of RX821002. Although these two experiments were not randomised, and so direct comparisons are not valid, this could suggest an increase in the sensitivity of α_2-adrenoceptors during anaesthesia. This is consistent with earlier reports of an increase in the efficacy of the α_2-adrenoceptor agonist, clonidine (Saunier *et al.*, 1993), during anaesthesia. As an adjunct in halothane anaesthesia, the ED_{50} of clonidine is 4.81 ± 0.8 μg / kg, whereas discontinuation of halothane results in a 5-fold increase in the ED_{50} of clonidine (25.48 ± 8.26 μg / kg) (Saunier *et al.*, 1993).

In Chapter 5, studies then progressed to investigate the effect of placement in the light / dark exploration box (LDEB) on a number of behavioural parameters in NK1+/+ and NK1-/- mice. The LDEB uses a novel environment as a non-noxious stressor to compare the ethological profile of behaviours that differ between NK1+/+ and NK1-/- mice (Stewart *et al.*, 2002; Fisher *et al.*, 2003; Fisher *et al.*, 2004; Herpfer *et al.*, 2005). Analysis of a number of behaviours is a particular strength of the LDEB protocol. Other LDEB models tend to oversimplify the behavioural response to the novel environment, by recording fewer behavioural responses and recording for a shorter duration of time (typically 3-4 min). Using the LDEB we have been able to demonstrate robust genotype-dependent differences in behaviour. The studies conducted here demonstrate that NK1-/- mice have:

- ***Higher*** *spontaneous locomotor activity*
- ***Higher*** *rearing behaviour*
- ***Fewer*** *risk assessment behaviours*
- *Spend* ***less*** *time in the light zone*

Herpfer *et al.*, (2005) demonstrated that all of the behaviours scored differed between genotype. However, for *number of returns* and *number of rears,* this was evident only after genotype-dependent differences in locomotor activity were taken into account, using ANCOVA analysis. In contrast to the studies performed by Herpfer *et al.*, (2005), which demonstrated an increase in the number of rears in NK1-/- mice compared to

NK1+/+ mice, following ANCOVA, ANCOVA abolished the genotype-dependent difference in this particular behaviour, in these current studies. This suggests the higher level of rearing behaviour observed in NK1-/- mice, is due to their higher locomotor activity compared to the NK1+/+ mice. However, the genotype-dependent differences in *risk assessment* behaviours and *time spent in the light zone* were still present after ANCOVA analysis. Therefore, these genotype-dependent behaviours were not due to a difference in locomotor behaviour between NK1+/+ and NK1-/- mice. Since behavioural arousal is intimately linked with activity of the LC-noradrenergic system (Smee *et al.*, 1975; De Sarro *et al.*, 1987) it is tempting to speculate that the higher locomotor behaviour in NK1-/- could be attributed to a difference in the regulation of the noradrenergic system between NK1+/+ and NK1-/- mice.

RX821002 was used to identify whether any genotype dependent behavioural differences could be attributed to a difference in noradrenergic transmission and, by implication, a difference in the α_2-adrenoceptors. Following systemic administration of RX821002 a number of behaviours differed in NK1+/+ mice. The following behaviours were changed by RX821002 in NK1+/+ mice only:

- *Time spent grooming in the light was* ***increased***
- *Latency to leave the light zone was* ***increased***
- *Time to return to the light zone was* ***decreased***
- *Number of rears in the dark was* ***decreased***
- *Number of returns to the light zone was* ***decreased***

With the exception of a decrease in the time to return to the light zone, and an increase in the latency to leave the light zone which are essentially a decrease in passive avoidance behaviour, these behavioural changes are generally consistent with an anxiogenic-like effect of RX821002. However, we have no comparable data pointing to the α_2-adrenoceptor mechanism whereby novelty leads to anxiety. A long held belief is that an increase in the activity of the LC system augments fearful or anxious responses (Redmond, Jr. & Huang, 1979). However, there is also evidence to suggest that an increase in the LC activity that accompanies anxious or stressful situations, is a compensatory, coping mechanism (Weiss *et al.*, 1994). Furthermore, pharmacological reports with α_2-adrenoceptor antagonists are quite inconsistent. They range from effects

of anxiogenesis (Handley & Mithani, 1984) to anxiolysis (La Marca S. & Dunn, 1994; Cole *et al.*, 1995). It is, therefore, possible that in these current studies potentiation of NA release following systemic administration of RX821002 is likely to produce 'fear-like' and perhaps 'anxiogenic-like' responses in our mice. Regardless of whether or not this interpretation of the 'emotional' significance of these behavioural changes is correct, that these behavioural effects, following pre-treatment with RX821002, are not observed in NK1-/- mice provides strong support for a dysregulation of the noradrenergic system in NK1-/- mice, perhaps attributable to α_2-adrenoceptors.

None of the genotype-dependent behavioural changes following RX821002 pre-treatment are shared by pre-treatment with DMI (Herpfer *et al.*, 2005). However, systemic administration of DMI resulted in a ***decrease* in total time spent grooming** (Herpfer *et al.*, 2005), whereas RX821002 resulted in an ***increase* in total time spent grooming**. The noradrenaline reuptake inhibitor DMI, and the α_2-adrenoceptor antagonist RX821002, have quite different mechanisms of action. It is possible that acute administration of DMI in the studies of Herpfer *et al.;* (2005) results in a decrease in extracellular NA, through a reduction in firing-dependent NA release (see: (Invernizzi & Garattini, 2004)). Administration of RX821002, via antagonism of α_2-adrenoceptors, is more likely to result in an increase in extracellular NA. Despite their different modes of action, it should be emphasised that both these compounds modify the behavioural response to the LDEB, of NK1+/+ and NK1-/- mice, in a genotype-dependent manner. However, the α_2-adrenoceptor antagonists, RX821002 and atipamezole, both decrease the *time to return to the light zone* following first exit, in NK1+/+ mice, only (Fisher *et al.*, 2003a; Fisher *et al.*, 2004b). The result is that NK1+/+ mice resemble NK1-/- mice pre-treated with either vehicle or an α_2-adrenoceptor antagonist. It may, therefore, be possible to link this particular behaviour to a difference in the regulation of the noradrenergic system between NK1+/+ and NK1-/- mice. This difference may be due to an attenuated response of the α_2-adrenoceptor in NK1-/- mice. The possibility that RX821002 and atipamezole act as antagonists at NK1 receptors, and so produce the same behavioural response that is observed following genetic disruption of the receptor, can be excluded. Binding studies have revealed that neither atipamezole nor RX821002 have any appreciable affinity (> 10 μM) for the NK1 receptor (Murray, Merck Sharp and Dohme-personal communication).

A number of behavioural changes following RX821002 (0.3 mg / kg i.p.) were observed in both NK1+/+ and NK1-/- mice. These included:

- *An overall **increase** in the total number of grooms*
- *An overall **increase** in the total time spent grooming*
- *An overall **increase** in the time spent in the light zone*
- *A **decrease** in the risk-assessment behaviours*, with the exception that *time spent in the flat back approach* was ***increased*** slightly.

This suggests retained activity of RX821002 in both genotypes, despite a decrease in the activity of α_2-adrenoceptors in NK1-/- mice. One possible explanation could be due to activation of post-synaptic adrenoceptors, which may be unchanged following genetic disruption of the NK1 receptor. Non-specific interactions with other neurotransmitter receptors, for example 5-$HT_{1A,}$ could also be a confounding factor.

Microdialysis performed in the LDEB (Chapter 5), has further demonstrated that following placement in the light novel compartment, NA efflux is increased by pre-treatment with RX821002 in NK1+/+ mice, only, over the same duration as the behavioural changes in the LDEB. However, the net increase in NA efflux, following RX821002 treatment, was not different, suggesting that the difference in the raw data is due to an underlying difference in the basal NA efflux.

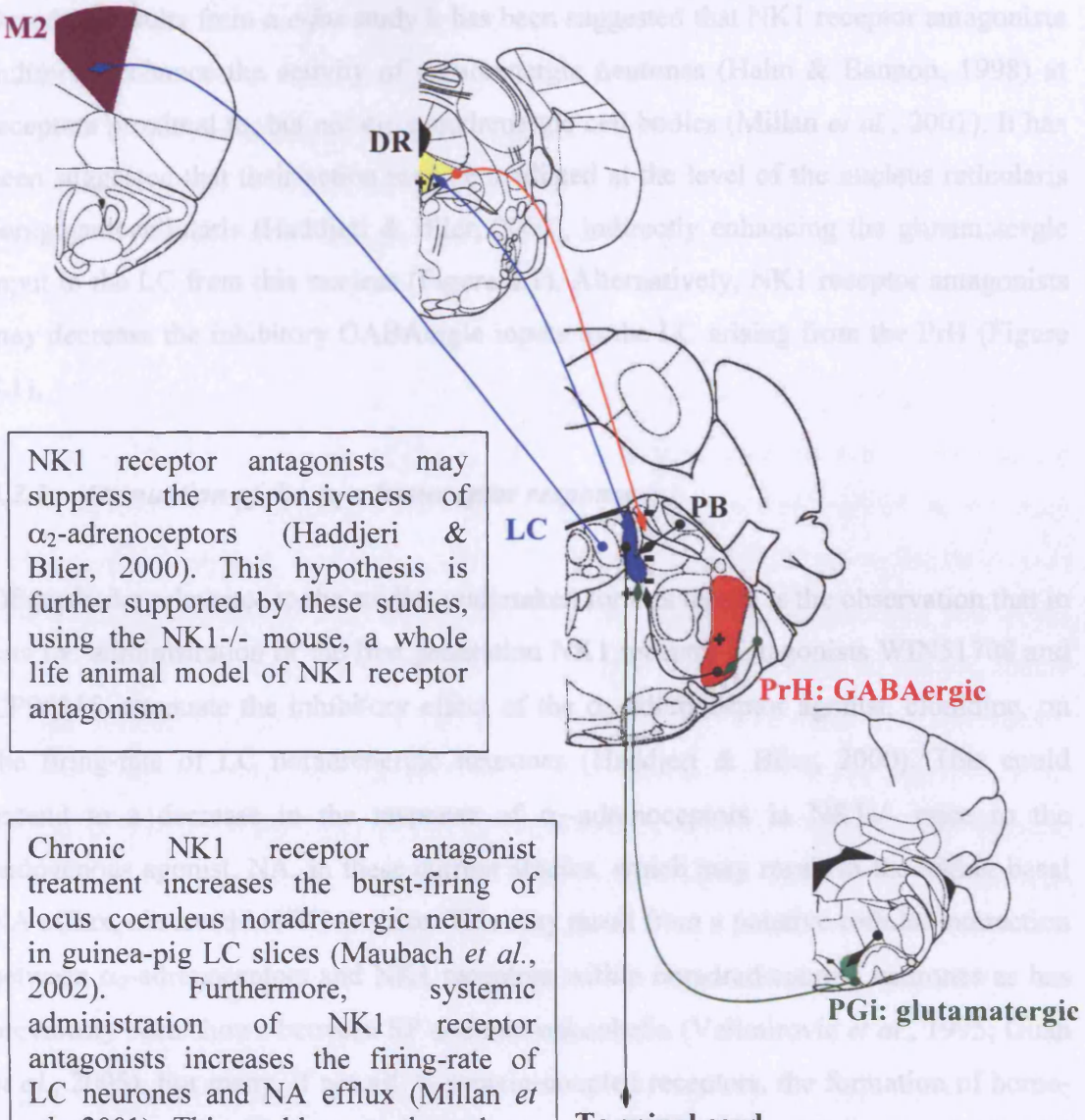
In Chapter 6, a molecular biological approach was adopted to investigate the prime candidates (α_2-adrenoceptors, noradrenaline transporters, dopamine-β-hydroxylase and tyrosine hydroxylase) that had been predicted to play an integral role in the difference in basal NA efflux in NK1-/- mice. The techniques used included:

- Immunohistochemical localisation of the above targets
- Western blot protein analysis of α_2-adrenoceptors and noradrenaline transporters in the LC and frontal cortex
- Autoradiographical analysis of the localisation and density of α_2-adrenoceptors
- Adrenaline-stimulated [^{35}S]GTPγS binding to provide a measure of the function of the α_2-adrenoceptors

However, none of the procedures used, provided conclusive evidence for a difference in any of the above targets (see caveats below).

8.2 WHY DOES GENETIC DISRUPTION OF THE EXCITATORY NK1 RECEPTOR INCREASE NORADRENALINE EFFLUX?

Previous studies have demonstrated that SP, through its actions at the NK1 receptor, increases the firing-rate of LC noradrenergic neurones (Guyenet & Aghajanian, 1977; Cheeseman *et al.*, 1983; Shen & North, 1992). Why does removal of this excitatory input to the LC result in an increase in NA efflux in NK1-/- mice also? Figure 8.1 demonstrates a general schema of how genetic disruption of the NK1 receptor could result in an increase in noradrenaline efflux and a lack of response to RX821002 in NK1-/- mice.



NK1 receptor antagonists may suppress the responsiveness of α_2-adrenoceptors (Haddjeri & Blier, 2000). This hypothesis is further supported by these studies, using the NK1-/- mouse, a whole life animal model of NK1 receptor antagonism.

Chronic NK1 receptor antagonist treatment increases the burst-firing of locus coeruleus noradrenergic neurones in guinea-pig LC slices (Maubach *et al.*, 2002). Furthermore, systemic administration of NK1 receptor antagonists increases the firing-rate of LC neurones and NA efflux (Millan *et al.*, 2001). This could occur through an action at NK1 receptors, which exert an indirect tonic inhibitory influence on locus coeruleus neurones, for example on an inhibitory input to PGi neurones or an excitatory input to PrH neurones. Therefore, it is possible that NK1 receptor antagonists unmask an excitatory input to the locus coeruleus in the presence of a stressor (Hahn & Bannon, 1999; Hahn & Bannon, 1998).

FIGURE 8.1: Possible mechanism by which genetic disruption of the NK1 receptor increases noradrenaline efflux. PB, parabrachial, LC, locus coeruleus, PGi, nucleus reticularis paragigantocellularis, PrH, nucleus prepositus hypoglossi, M2, frontal cortex, M2 region, DR, dorsal raphé.

Based on results from a *c-fos* study it has been suggested that NK1 receptor antagonists indirectly enhance the activity of noradrenergic neurones (Hahn & Bannon, 1998) at receptors proximal to, but not on, noradrenergic cell bodies (Millan *et al.*, 2001). It has been suggested that their action may be mediated at the level of the nucleus reticularis paragigantocellularis (Haddjeri & Blier, 2000), indirectly enhancing the glutamatergic input to the LC from this nucleus (Figure 8.1). Alternatively, NK1 receptor antagonists may decrease the inhibitory GABAergic inputs to the LC arising from the PrH (Figure 8.1).

8.2.1 Attenuation of the α_2-adrenoceptor response

Of particular relevance to the studies undertaken for this thesis, is the observation that in rats i.v. administration of the first generation NK1 receptor antagonists WIN51708 and CP96345, attenuate the inhibitory effect of the α_2-adrenoceptor agonist, clonidine, on the firing-rate of LC noradrenergic neurones (Haddjeri & Blier, 2000). This could extend to a decrease in the response of α_2-adrenoceptors in NK1-/- mice to the endogenous agonist, NA, in these current studies, which may result in the higher basal NA efflux, observed in NK1-/- mice. This may result from a putative cellular interaction between α_2-adrenoceptors and NK1 receptors within noradradrenergic neurones as has previously been shown between SP and met-enkephalin (Velimirovic *et al.*, 1995; Guan *et al.*, 2005). For many, if not all, G protein-coupled receptors, the formation of homo- and heterodimeric receptors seems to be a general principle (see: (Pfeiffer *et al.*, 2003)). A major prerequisite for the physiological assembly of heterodimeric GPCRs *in vivo* is their coexpression in the same cell. NK1 receptors (Nakaya *et al.*, 1994), α_2-adrenoceptors (Scheinin *et al.*, 1994) and μ opioid (Mansour *et al.*, 1994; Van Bockstaele *et al.*, 1996a) receptors are all expressed in the LC. Furthermore, the μ opioid receptor forms stable heterodimers with the NK1 receptor (Pfeiffer *et al.*, 2003) and α_{2a}-adrenoceptor (Jordan *et al.*, 2003). The μ opioid-NK1 heterodimerization results in an alteration in the trafficking and resensitisation profile of the μ opioid receptor (Pfeiffer *et al.*, 2003) through delayed recycling and resensitisation kinetics. Interestingly, the rewarding effects of morphine are absent in mice lacking the NK1 receptor (Murtra *et al.*, 2000; Ripley *et al.*, 2002) and increased NK1 receptor stimulation in mice lacking NA leads to reduced opioid efficacy (Jasmin *et al.*, 2002), demonstrating an intimate relationship between these receptors. Therefore, a

developmental consequence of disrupting expression of the NK1 receptor, may be an increase in the physical association (cross-talk) of μ opioid and α_2-adrenoceptors resulting in an attenuation of their signalling properties. Alternatively, there may be a cross-talk between α_2-adrenoceptors and NK1 receptors which is lost by genetic disruption of the NK1 receptor and this may attenuate signalling through the α_2-adrenoceptor in NK1-/- mice.

Alternatively, a result of genetic disruption of the NK1 receptor may be an alteration in the trafficking of the α_2-adrenoceptor to the cell surface membrane. LDCVs, are Golgi-derived neuropeptide-rich units that belong to the 'regulated' part of the secretory pathway (see:(Julius & Basbaum, 2005)). LDCVs mediate both neuropeptide release and trafficking of receptors to the pre-synaptic cell surface. It is possible, that in the absence of the NK1 receptor, α_2-adrenoceptors are no longer targeted to the 'regulated' protein secretory pathway, but rather become part of the ubiquitous or 'constitutive' pathway, which carries housekeeping proteins to the cell surface. For example, in an analogous manner, it has recently been demonstrated that the trafficking of the δ-opioid receptor to large dense-core vesicles (LDCVs) depends on its physical interaction with the SP domain of protachykinin, the precursor of SP (Guan *et al.*, 2005). In the absence of SP in preprotachykinin A (PPT-A) mice, targeting of the δ-opioid receptor to the cell surface is mediated through the 'constitutive' secretory pathway. The functional significance of these two pathways is demonstrated by PPT-A knockout mice. The analgesic action of δ-opioid agonists is believed to arise through inhibition of Ca^{2+} channels mediated by the δ-opioid receptor. The analgesic action of a δ-opioid agonist completely disappears in PPT-A knockout mice, suggesting that these δ-opioid receptors, arising from the 'constitutive' pathway, are not functional or are not coupled to the Ca^{2+} channels in the same way that δ-opioid receptors arising from the 'regulated' pathway are (Guan *et al.*, 2005). This could extend to the activity of the α_2-adrenoceptor in NK1-/- mice. Genetic disruption of the NK1 receptor could result in targeting of α_2-adrenoceptors to the 'constitutive' pathway, leading to a decrease in the inhibition of Ca^{2+} channels and consequently an increase in NA efflux and a decrease in the inhibitory activity of these receptors. Furthermore, immunocytochemistry can underestimate the contribution of the 'constitutive' pathway to plasma membrane receptor localisation (Guan *et al.*, 2005). Thus a change in the receptor trafficking may

have occurred in NK1-/- mice, at least in those neurones that would have expressed NK1 receptors.

8.2.2 Decreased activation of the inwardly rectifying potassium channel

Stimulation of G protein-coupled receptors coupled to $G_{i/o}$ proteins, leads to inhibition of adenylyl cyclase by the α subunit of the heterotrimeric G proteins, as well as inhibition of voltage-dependent Ca^{2+} channels and activation of inwardly rectifying potassium channels (GIRKs) directly by the βγ dimers (Reuveny *et al.*, 1994; Huang *et al.*, 1995) in a membrane associated process (Williams *et al.*, 1988; Miyake *et al.*, 1989). Genetic disruption of the NK1 receptor may result in a change in the biophysical properties of LC neurones. Decreased hyperpolarisations, perhaps due to a decrease in the activation of GIRKs, via attenuated signalling through the α_2-adrenoceptor would mean that LC neurones display hyperpolarisations of shorter duration and are, therefore, more excitable. Alternatively, there may be a change, perhaps a decrease, in Ca^{2+}-activated K^+ conductances, as has been postulated previously (Maubach *et al.*, 2002).

8.3 CAVEATS

Placement of rats in a light novel environment results in an increase in NA efflux (Dalley & Stanford, 1995; Dalley *et al.*, 1996; McQuade *et al.*, 1999). One set of experiments was, therefore, aimed at determining whether there are differences in NA efflux in NK1+/+ and NK1-/- mice on exposure to a novel environment, and whether or not NA efflux can be modified in a genotype dependent fashion (Chapter 5). If so this would provide further support for a difference in the regulation of noradrenergic neurotransmission between NK1+/+ and NK1-/- mice.

Analysis of raw microdialysis data demonstrated that NA efflux was increased only in RX821002 pre-treated NK1+/+ mice, when placed in the novel environment. However, analysis of net data did not show a difference in the incremental increase in NA efflux in vehicle or RX821002 pre-treated NK1+/+ and NK1-/- mice. This suggests that the higher basal NA efflux observed in NK1+/+ destined for treatment with RX821002 was responsible for the observed effect of RX821002 in the raw data. It is possible that increasing the sample sizes in this experiment would lead to a net increase in NA efflux

that is highest in NK1+/+ mice pre-treated with RX821002. However, performing Mead's (1988) 'Resource Equation' (see: Festing *et al.*, 2002) demonstrates that a difference should be apparent, should one exist.

In Chapter 6 no difference was found in the density of α_2-adrenoceptors between NK1+/+ and NK1-/- mice using [^{3}H]RX821002 autoradiography. It is possible that an insufficient number of animals (n = 3) was used to reveal any differences. This may also be the case concerning the adrenaline-stimulated [^{35}S]GTγS binding assay, used to provide a measure of the G-protein coupling of the α_2-adrenoceptors. Although there was a general trend towards lower functional activity of α_2-adrenoceptors in NK1-/- mice compared with NK1+/+ mice, the variable nature of the technique may have masked a very small change. The variable nature of the technique, which has been noted by others (Newman-Tancredi *et al.*, 2000), may, therefore, require additional animals to provide a conclusive answer as to the functional state of the α_2-adrenoceptors in NK1+/+ and NK1-/- mice.

8.4 PROPOSALS FOR FUTURE EXPERIMENTS

- Examine NA efflux in wildtype mice, following pharmacological manipulation of NK1 receptors. This could be achieved by chronic, or local administration into targeted brain areas, of GR205171, the NK1 receptor antagonist most suitable for use in rats and mice ($pK_i = 9.5$, (Gardner *et al.*, 1996)). We would predict that chronic administration of GR205171 would result in an increase in NA efflux.

- Investigate the behavioural, electrophysiological and neurochemical profiles of a conditional NK1 receptor knock out mouse. Using the cre-lox P mediated method of receptor deletion (under the control of either the tyrosine hydroxylase or noradrenaline transporter promoter) it would be possible to examine the effects of selective genetic disruption of NK1 receptors expressed in LC neurones. Selective disruption of NK1 receptors expressed by noradrenergic LC neurones would help to identify whether NK1 receptor antagonists exert an excitatory effect through an indirect action.

- Examine the electrophysiological activity of the LC in NK1+/+ and NK1-/- mice, in particular focusing on the GIRKs. Although the firing-rate of DR neurones in NK1+/+ and NK1-/- mice has been investigated, no studies have profiled the activity of the LC in these animals. *In vivo* LC neurones fire tonically (1 to 2 Hz), with their rate of firing closely correlated to the state of arousal of the animal. However, in an *in vitro* preparation, where all of the major inputs to the LC have been severed, they also show a similar rate of firing compared to the *in vivo* situation (Nestler *et al.*, 1999), suggesting a pacemaker activity. It is possible that this pacemaker activity is different between the two genotypes, or has a different response to anaesthesia. This may underlie the difference in basal NA efflux observed between NK1+/+ and NK1-/- mice during halothane anaesthesia.

- Investigate the possibility of a cellular interaction (formation of heterodimers) between α_2-adrenoceptors and NK1 receptors using coimmunoprecipitation followed by Western blot protein analysis. Such a physical interaction could provide a plausible explanation for cross-talk between NK1 receptors, α_2-adrenoceptors or μ opioid receptors. This physical interaction may result in an altered internalization or desensitization profile of the receptors, or an alteration in the mechanisms and functional consequences of downstream signalling.

- Microdialysis and synaptosomal studies have provided evidence for a DMI-resistant, fluoxetine-sensitive uptake site in the rat cortex (Hughes & Stanford, 1998; Hughes & Stanford, 1996). The lack of effect of cortical infusion of DMI in NK1-/- mice (See: Chapter 3) may be due to an upregulation of this particular binding site. Therefore, future studies could focus on the DMI and fluoxetine induced inhibition of [^{3}H]NA uptake into cortical synaptosomes, following a noradrenergic neuronal lesion using N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4). This would provide *in vitro* evidence for the presence of an alternative uptake site, which may differ between the two genotypes.

APPENDIX I

GENOTYPING

- 5mm of tail tip is removed using a clean razor blade and placed into a sterile 1.5 ml eppendorf tube.
- 750 µl of lysis buffer (Tail lysis buffer: 50 mM Tris pH 8 (Sigma) 100 mM EDTA pH 8 (Sigma) 10 mM NaCl (BDH) 1% SDS (Sigma) and 22.25µl Proteinase K (20mg/ml; Sigma) is added to each sample and vortexed briefly. Samples are then incubated overnight at 55°C, briefly vortexing two to three more times. If necessary, store at -20°C.
- If necessary defrost samples. Vortex briefly, then centrifuge for 3 min at 13000 rpm. The supernatant is then transferred to clean 1.5 ml microfuge tubes and 200 µl protein precipitation solution (Puregene, Minneapolis, USA) is added. Vortex the samples for 20 s and centrifuge for 3 min at 13 000 rpm.
- The supernatant is then removed and the pellet washed in 300 µl 70 % ethanol. Then centrifuged for 3 min at 13 000 rpm.
- The supernatant is removed and the tubes left open to dry to allow the ethanol to evaporate.
- 100 µl of TE (10mM Tris pH 8 (Sigma), 1 mM EDTA pH 8 (Sigma) is added to each sample. Samples can then be stored at 4 °C.
- Make up reaction mix:

Ultra pure water	32.5 µl
Taq DNA polymerase 10 X reaction buffer (Promega, Southampton,UK)	5.00 µl
25 mM $MgCl_2$ (Promega)	3.00 µl
10µM dATP (Promega)	0.25 µl
10µM dCTP (Promega)	0.25 µl
10µM dGTP (Promega)	0.25 µl
10 µM dTTP (Promega)	0.25 µl
NeoF[1] (0.5 µg/µl; Sigma Genosys, Cambridge, UK)	1.00 µl
NK1-F[2] (0.5µg/µl; Sigma Genosys)	1.00 µl
NK1-R[3] (0.5µ/ul; Sigma Genosys)	1.00 µl
Taq DNA polymerase (Promega)	0.50 µl
DNA sample	5.00 µl
Total	**50.00 µl**

[1] NeoF: 5'-GCAGCGATCGCCTTCTATC-3'
[2] NK1-F: 5'-CTGTGGACTCTAATCTCTTCC-3'
[3] NK1-R: 5'-ACAGCTGTCATGGAGTAGATAC-3'

- Tubes are placed in a PCR temperature cycler (PTC-100 Programmable Thermal Controller, MJ Research, Boston, USA) and the following programme run:

95 °C 5 min

60 °C 30 s	
72 °C 30 s	
94 °C 30s	x 34
60 °C 30s	

72 °C 5 min

- Add 5 µl loading buffer (0.25 % bromophenol blue (Sigma); 0.25 % xyelene cyanol FF (Sigma); 30 % glycerol (BDH)) to each sample and mix thoroughly.
- Run 11 µl of each sample on a 2 % agarose gel in TBE buffer (National Diagnostics, Hull, UK) containing 10 µg / ml ethidium bromide (Sigma) at 120 mV for approximately 1 h.
- Visualise and photograph under ultraviolet transillumination

APPENDIX II

Protocol 2.8.1 Immunohistochemistry with chromogenic detection

Reactions were carried out by gently agitating sections on a tissue rocker at room temperature.

- Sections were first rinsed in 0.1M PB in 1.5 ml eppendorf tubes
- Placed in 3% normal goat serum (NGS) in TTBS to block non-specific binding. 2 % H_2O_2 was also included to block endogenous peroxidase activity. Sections left on rocker for 1 h[1].
- Incubate sections in primary antibody in NGS on a rocker for 2-3 d at 4°C.
- Sections washed three times for 10 min in 0.1M PB.
- Sections incubated in biotinylated goat anti-rabbit IgG (1:500 in TTBS; Vector Laboratories) for 2 h[2].
- Sections washed three times for 10 min is 0.1M PB.
- Add avidin-peroxidase solution (0.5 % Reagent A and 0.5 % Reagent B in TTBS; Vectastain Elite ABC kit [Standard]; Vector laboratories) for 1 h.
- Sections washed three times for 20 min in 0.1 M PB.
- Sections washed briefly in 0.15 M Tris buffer.
- Sections are then developed using a DAB substrate kit for peroxidase (Vector)
- Reaction is stopped by washing sections twice for 5 min each in 0.1M PB.
- Sections are then rinsed briefly in 0.01M PB.
- Sections mounted onto gelatine-coated slides and left to air dry.
- Sections are then dehydrated in ethanol solutions of increasing strength (2 min in distilled water, 70 % ethanol x 2; 95 % ethanol x 2; 100 % ethanol x 2; 2 min in each).
- Clear in histoclear (2 min x 2; National Diagnostics).
- Slides coverslipped using DPX mounting medium (BDH).

[1] The blocking serum should be that of the animal in which the secondary antibody is raised.

[2] The secondary antibody must be raised against the animal in which the primary antibody was raised.

APPENDIX III

Protocol 2.8.2 Fluorescence Protocol

- Sections placed in 1.5 ml Eppendorf tubes in 0.1M PB.
- Washed on rocker for 10 min.
- Block for 1 h to reduced non-specific binding in 3 % Normal Donkey/Goat serum in TTBs. The serum used must be that of the animal in which the secondary antibody was raised.
- Incubate sections in primary antibody for 2-3 days at 4°C or over night at room temperature.
- Sections washed three times for 10 min each in 0.1 M PB.
- Sections incubated in biotinylated secondary antibody for 2 h (1:200 in NGS; Vector Laboratories).
- Sections washed three times for 10 min each in 0.1M PB.
- Sections incubated in dark in Flourescein Avidin D (1:4000 in TTBs; Jackson Cy3) for 45 min.
- All sections were then removed washed three times for 10 min in 0.1 M PB.
- Sections subsequently mounted in 0.01 M PB and left to dry in the dark.
- Slides were coverslipped in antifade kit.

APPENDIX IV

Protocol 2.8.3 TSA indirect amplification method with FITC fluorescence

- Sections placed into 1.5 ml eppendorfs with 0.1M PB.
- Sections blocked in 3 % normal goat serum and 0.3 % Triton X-100 in 0.1M PB for 1 h.
- Sections incubated in primary antibody overnight at room temperature or 2-3 d at 4°C.
- Sections washed three times for 10 min each in 0.1M PB
- Sections incubated in secondary antibody biotinylated goat anti-rabbit IgG (1:200 in TTBs) for 90 min.
- Sections washed three times for 10 min each in 0.1M PB. Meanwhile make up the avidin-peroxidase solution (0.4 % Reagent A and 0.4 % Reagent B in TTBs; Vectastain Elite ABC Kit [Standard]; Vector)
- Sections washed three times for 10 min each in 0.1M PB.
- Sections incubated in avidin-peroxidase solution for 30 min. Meanwhile, remove biotinylated tyramide solution from the fridge and leave to thaw.
- Sections washed three times for 10 min each in 0.1M PB.
- Sections incubated in Biotinylated tyramide (1:75 in diluent; TSA^{TM} Biotin System; NEN Life Science Products, Boston, USA) for 7 min.
- Sections washed three times for 10 min each in 0.1M PB
- Sections incubated in the dark in Fluorescein Avidin D (1:600 in 0.1M PB; Vector Laboratories) for 2 h.
- Sections washed three times for 10 min each in 0.1M PB.
- Sections mounted, in 0.01M PB, onto Superfrost Plus Microscope Slides (BDH) and left to dry in the dark.
- Slides coverslipped with Citifluor (Citifluor, London, UK) or Prolong® Antifade Kit (Molecular Probes, Leiden, The Netherlands).

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